Intramural Activities Volume 1

October 1, 1988–September 31, 1989

89 annual report

Division Of

Cancer Etiology

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
National Institutes of Health

NATIONAL CANCER INSTITUTE

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NATIONAL CANCER INSTITUTE (U.S.)

ANNUAL REPORT

DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 through September 30, 1989

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ANNUAL REPORT
OF THE
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

Richard H. Adamson, Ph.D., Director

October 1, 1988 through September 30, 1989

OVERVIEW

The Division of Cancer Etiology (DCE) is comprised of three major programs: the Biological Carcinogenesis Program, the Chemical and Physical Carcinogenesis Program, and the Epidemiology and Biostatistics Program. The Biological Carcinogenesis Program consists of one extramural component (the Biological Carcinogenesis Branch) and six intramural laboratories (the Laboratory of Cellular and Molecular Biology, the Laboratory of Molecular Oncology, the Laboratory of Molecular Virology, the Laboratory of Tumor Virus Biology, the Laboratory of Viral Carcinogenesis, and the Laboratory of Tumor Cell Biology). The Chemical and Physical Carcinogenesis Program consists of two extramural components (the Chemical and Physical Carcinogenesis Branch and the Radiation Effects Branch) and eight intramural laboratories (the Laboratory of Biology, the Laboratory of Cellular Carcinogenesis and Tumor Promotion, the Laboratory of Chemoprevention, the Laboratory of Comparative Carcinogenesis, the Laboratory of Experimental Carcinogenesis, the Laboratory of Experimental Pathology, the Laboratory of Human Carcinogenesis, and the Laboratory of Molecular Carcinogenesis). The Epidemiology and Biostatistics Program consists of one extramural component (the Extramural Programs Branch) and four intramural branches (the Biostatistics Branch, the Clinical Epidemiology Branch, the Environmental Epidemiology Branch, and the Radiation Epidemiology Branch).

The Division has been structured in such a way as to maximize interaction between intramural scientists and the extramural community, facilitate the sharing of scarce resources and generally improve the management of research in cancer etiology. Efforts continue to further increase interaction between the biological carcinogenesis and chemical carcinogenesis laboratories and to enhance their interaction with the components of the Epidemiology and Biostatistics Program. The current organizational chart for DCE is shown in Figure 1. Two years ago an Associate Director for Biological Carcinogenesis joined the DCE staff. He is currently also serving as Acting Chief, Laboratory of Molecular Virology.

The distribution of funds among the intramural and extramural components of the Chemical and Physical Carcinogenesis Program, the Biological Carcinogenesis Program, and the Epidemiology and Biostatistics Program is shown in Table 1 and Figure 2.

The past year has seen a continued reduction in overall contract support. This has been realized by reducing activities which provide materials and services, and by initiating and continuing various cost-recovery mechanisms. For example, in the Biological Carcinogenesis Branch four resource contracts

are functioning in the cost-recovery, or "payback" mode. These include one for production of viral reagents, one for supplying animals, one for specialized testing services, and one for storage and distribution of stored frozen biological reagents. In the Chemical and Physical Carcinogenesis Branch, payback systems have been established for the Radiochemical Repository and for the Chemical Carcinogen Reference Standard Repository. All samples distributed under the chemical research resource program are now under this cost-recovery system. Reimbursement for full or partial costs of services has led to a more careful use of costly resource reagents and chemicals, with a subsequent reduced level of effort in several resource contracts or the termination of unnecessary activities. As support for research contracts has decreased, support for investigator-initiated research grants has continued to increase, and the Cooperative Agreement is now being utilized as an additional instrument of support. In addition to its intramural and extramural research, the Division has been involved in other activities during the past year which merit attention. These are:

1. Frederick Cancer Research Facility (FCRF)

   Research conducted by the contractor began in the early 1970s as a series of unrelated tasks or projects. Today, the carcinogenesis program at the FCRF has gradually evolved into a coordinated effort on the mechanisms of action of viral and chemical carcinogens. FCRF is the focus of NCI research on acquired immunodeficiency syndrome (AIDS), with particular emphasis on the development of an AIDS vaccine. As a separate effort, the contractor provides support services and materials to intramural investigators who work in NCI laboratories at the Frederick facility. Government and contractor scientists have combined their efforts to create a center of excellence for cancer research. At the present time the following DCE laboratories are located at FCRF: the Laboratory of Comparative Carcinogenesis, the Laboratory of Molecular Oncology, the Laboratory of Viral Carcinogenesis and a section of the Laboratory of Tumor Cell Biology.

2. The DCE Board of Scientific Counselors

   Chartered in 1978, the Board is an advisory body whose members are drawn from the scientific community outside NIH. Chosen for their recognized expertise in chemical carcinogenesis, molecular biology, viral oncology, chemoprevention, epidemiology, immunology, pathology, and genetics, the members of the Board advise the Division Director on a wide variety of matters which concern the progress of the Division's programs. One particularly important responsibility of the Board has been the examination of the productivity and performance of staff scientists by site visit review of the intramural laboratories and branches. These visits have been conducted by teams which, as a rule, are comprised of two to four members of the Board and four or more investigators from the scientific community outside NIH whose special fields of expertise match those of the scientists in the Laboratory or Branch being reviewed. The site visit reports, which reflect a consensus of the members of the team, are critically examined by

the entire Board of Scientific Counselors and, after discussion, recommendations based on the reports are submitted to the Division Director. Approximately one year later the Division Director reports back to the Board on the changes made as a result of the site visit.

The third cycle of site visits to the Division's intramural operation began in October 1987. Since that time, the following Laboratories and Branches have been site visited: Biostatistics Branch, Laboratory of Tumor Virus Biology, Laboratory of Viral Carcinogenesis, Environmental Epidemiology Branch, Laboratory of Cellular and Molecular Biology, and Clinical Epidemiology Branch. Site visits will also be made to the Laboratory of Molecular Carcinogenesis, Laboratory of Biology, and Laboratory of Comparative Carcinogenesis in the coming year.

Another important function of the Board is that of concept review, in which the members pass judgment on concepts for grant- or contract-supported activities. The Board continued to examine new concepts this year as new directions in extramural research activities developed.

Several workshops, involving Board members as well as participants from the scientific community outside NIH, were held this year. One workshop held during the past year was entitled "Mechanisms of Pathogenic Diversity of Epstein-Barr Virus" which resulted in the RFA "New Approaches to Studying Epstein-Barr Virus Oncogenesis." Other workshops held this year included "International Workshop on Pathogenesis and Prevention of Hepatocellular Carcinoma," "Interactions of T-Antigens with Proto-Oncogenes," and "Retroviruses and Human Disease: Search for New Agents."

As a result of such workshops, several request for applications (RFAs) have been funded during the past year, including "Animal Models for Human Papillomavirus-Associated Neoplastic Diseases," "Retrovirus Animal Models and HIV Pathogenesis," and "Epidemiologic Studies of HIV-Associated Malignancies."

In addition, the Division has assumed responsibility for three RFAs which were initiated by the organ systems staff in DCPC prior to the reorganization of the program. The three RFAs are "In Vitro Transformation of Human and Animal Mammary Epithelial Cells by Chemical Carcinogens," "Mechanisms of Alcohol and Tobacco-Related Carcinogenesis of the Oral Cavity," and "Identification of Genetic Alterations Involved in Bladder Carcinogenesis."

The Small Grants Program for Cancer Epidemiology was reannounced in August 1988 following intensive evaluation and approval by the DCE Board of Scientific Counselors. The program is serving a useful purpose especially for young investigators, recruiting doctoral students, fellows, and junior faculty into cancer epidemiology. Several recent changes increase the flexibility of the grants: allowable direct costs increased to $50,000 and the maximum project period increased to three years. Competitive

renewal is permitted. The amended purposes of the program, all relevant to cancer epidemiology, include: planning a complex study; developing or validating a laboratory or statistical procedure; obtaining rapid funding; analyzing previously collected data, including meta-analysis; and resolving problems of methodology. The Small Grants Program is an important innovation and the quality of applications continues to improve.

The objectives of the extramural research programs are accomplished through a variety of extramural support mechanisms including traditional individual research project grants (R01), program project grants (P01), First Independent Research Support and Transition (FIRST) awards (R29), conference grants (R13), Cooperative Agreements (U01), contracts (N01), small business innovative research (SBIR) contracts (N43/44) and grants (R43/44), academic research enhancement awards (R15), outstanding investigator awards (R35) and the new Method to Extend Research In Time (MERIT) award (R37).

Continuing modifications of funding mechanisms approved by the Board include a gradual transfer of current resources to a cost-reimbursement system (payback system), increased availability of funding to support new resource activities, the phasing out of contract-supported research in areas adequately covered by grant applications, use of RFAs to stimulate research activity in high priority areas, and the use of the Cooperative Agreement as an additional mechanism of support for investigator-initiated research involving significant involvement of NCI staff.

The Division is most grateful to the members of the Board for giving so generously of their time, effort, and expertise in helping to guide the future of the Division's programs. There is every expectation that the Board will continue to play a vital role as the Division's foremost outside advisory group.

TABLE I

NATIONAL CANCER INSTITUTE

DIVISION OF CANCER ETIOLOGY

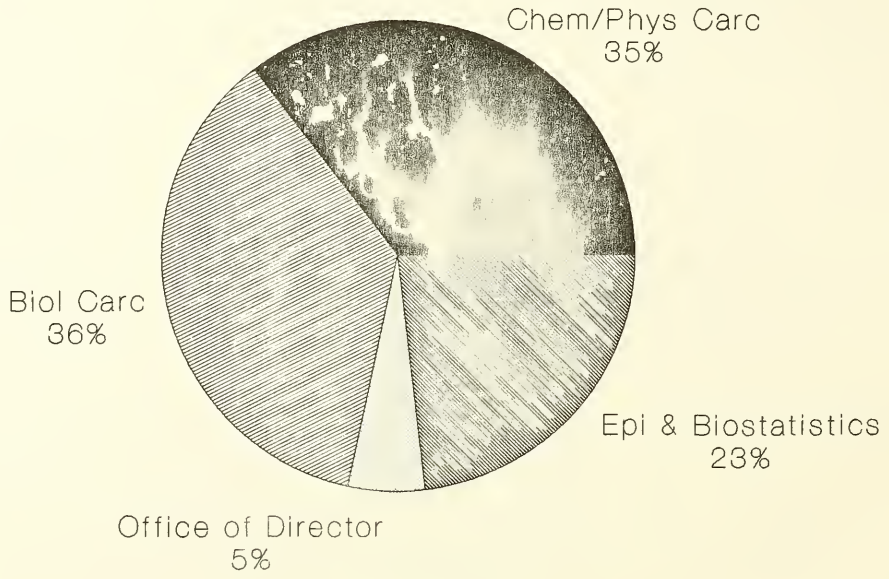
Table of Mechanisms by Organizational Unit Based On
Estimated Current Level of Expenditures
(Dollars in Thousands)

FY 1989 Estimate

	Office of the Division Director	Chemical and Physical Carcinogenesis	Biological Carcinogenesis	Epidemiology and Biostatistics	Total
In House	13,502	20,892	21,637	9,617	65,648
Contracts	2,647	4,750	4,126	24,153	35,676
RFA	0	2,706	3,875	2,313	8,894
Cooperative Agreements	828	3,101	0	1,352	5,281
Research Project Grants	0	82,137	86,188	36,710	205,035
TOTAL	16,977	113,586	115,826	74,145	320,534

Figure 2

NATIONAL CANCER INSTITUTE
DIVISION OF CANCER ETIOLOGY



Distribution of Funds, FY 1989 Estimate

SCIENTIFIC HIGHLIGHTS

Introduction: The Division of Cancer Etiology is responsible for planning and conducting the Institute's program of coordinated research on cancer causation and basic research on prevention. It is also responsible for directing and coordinating AIDS vaccine research and development efforts for NCI. The Division supports both intramural laboratories and branches and extramural programs which seek to elucidate the mechanisms of cancer induction at each step of the cellular process from initiation to transformation of normal cells into malignant cells. The overall purpose of these studies is to provide information for reversing, interrupting, or preventing this process before the development of clinical disease.

To accomplish these goals, investigators pursue fundamental studies on normal and malignant cells and on carcinogens or procarcinogens, such as viruses and chemicals, using the disciplines of cellular and molecular biology, immunology, biochemistry, and microbiology. Epidemiologic studies of human populations are performed to identify risk factors predisposing to various cancers using the disciplines of clinical medicine, genetics, mathematics, biometry, and biostatistics.

Fundamental information on biological carcinogenesis has been acquired by studying human and animal tumor viruses which induce cells to become malignant. These viruses provide tools for understanding how cellular genes and their products convert a normal cell into a tumor cell. Of equal importance, horizontally transmitted particulate human viruses may themselves be directly responsible for some malignant transformations in man. Investigations of biological carcinogenesis have shown that viral information present as nucleic acid sequences in the genes of normal cells and replicating with them may be intimately involved in the development of cancer. This may occur through either the direct effects of viral transforming genes, through the influence of viral enhancing elements (promotors or long terminal repeats) on cellular oncogenes, or through the interaction of viruses with a variety of environmental factors, such as hormones, chemicals, radiation, and the like.

Similarly, chemical carcinogens, both naturally occurring and synthetic, provide a diverse group of chemicals with cellular and tissue selectivities that influence transformation and progression from the normal to the malignant state. Studies in the area of chemical/physical carcinogenesis cover a broad range of approaches with emphasis on the mechanisms of action of chemical and physical agents. Investigators have focused on the effects of carcinogens on cell structure and function, the relationships between molecular structure and carcinogenic activity, enzyme systems associated with the metabolic activation of procarcinogens to carcinogenic forms, the effects of the binding of carcinogens to DNA, systems that repair DNA damage, and on the possible activation of oncogenes by chemical carcinogens. Other relevant efforts include investigation of the role of various factors in the environment of the cell; for example, promoters, hormones and growth factors which may be required for the progression of the "initiated" cell to the malignant state. Finally, studies on the natural history of cancer in humans and on the incidence of cancers in different geographic locations help to identify causal associations of intrinsic and extrinsic risk factors with various cancers. In view of the increasing importance of nutrition and life-style in the causation

or prevention of cancer, in particular the role of macro- and micronutrients in the diet, alcohol consumption and tobacco use, special emphasis has been given to projects that have immediate health implications. Many studies deal with the presence of mutagens, carcinogens and natural anticarcinogens in foods; other studies assess the carcinogenic effects of smokeless tobacco and passive smoking. In addition, major studies on the viral etiology of cancer, cancer incidence in the workplace, effects of low-level radiation including radon, and environmental pollutants in air, water, and soil are under investigation. Studies on the pathogenesis, natural history, transmission, and cofactors for HIV (human immunodeficiency virus) infection have received major emphasis during the past year, as has HIV-associated malignancies.

BIOLOGICAL CARCINOGENESIS PROGRAM

During the past fiscal year the Biological Carcinogenesis Program continued to make research advances in the study of RNA and DNA viruses, growth factors and oncogenes. Some of these studies are highlighted below.

RNA VIRUS STUDIES

Human Immunodeficency Virus (HIV)

Spindle-like cells were successfully cultured for long periods of time from tissue specimens taken from the lung, mouth, skin, orbit, tonsil, and pleural effusion obtained from acquired immunodeficiency syndrome (AIDS) patients with disseminated Kaposi's sarcoma. Functional studies of these cells with respect to their role in matrix formation or destruction with the help of collagenases, chemotactic and chemoinvasion capabilities have just been completed. The apparent conclusion of this study seems to be that these cultured cells are abnormal smooth muscle cells of vascular origin. This cell system can be successfully used for virus isolation in situations when the T-cell system fails.

Several drugs have been examined either alone or in combination for inhibition of HIV-1 replication in cell cultures. Drugs that inhibit virus replication by mechanisms other than reverse transcriptase inhibition and DNA chain termination may be useful in the treatment of patients with AIDS and AIDS-related complex (ARC).

Several approaches to the development of an AIDS vaccine are being pursued. The potential usefulness of synthetic peptides as candidate AIDS vaccines has been explored, especially a peptide generated from the p17 sequence on the surface of HIV-1. Because of the genetic variability in the gp120 region of different HIV-1 isolates, which show as much as 20% divergence in the amino acid sequence of gp120, it is important to look for approaches other than the conventional aim of a gp120 vaccine in the development of an AIDS vaccine.

Results of a six-year prospective study of a cohort of HIV-1-seropositive homosexual men have shown that neutralizing antibodies are correlated with long periods of disease-free status. Using a monoclonal antibody an HIV neutralizing epitope has been mapped to a 24-amino acid region within the viral envelope. Others, using alternate techniques, have mapped the same site, now recognized as a major type-specific immunodominant epitope.

Human T-cell Lymphotropic Virus Type I (HTLV-I)

HTLV-I has been studied as the etiologic agent of adult T-cell leukemia. Studies have shown that trans-activation of HTLV-I long terminal repeat (LTR) by the virus-coded trans-activating protein Taxl is correlated with the presence of a cAMP-responsive octonucleotide. It has also been established that two signal transduction agents, cAMP and TPAl, are both potent activators of the HTLV-I LTR. The cAMP-responsive and the TPA-responsive sequences have been shown to be the HTLV-I LTR.

It has been shown that the Taxl protein binds specifically to a zinc affinity column. The putative metal binding domain of Taxl may play an important role in trans-activation. Since Taxl appears not to bind DNA directly, its interaction with cellular transcription factors may be facilitated by coordinating a common zinc ion.

Three lines of transgenic mice expressing the HTLV-I taxl gene were previously developed here; these mice develop neurofibromas that recruit granulocytes as a result of tumor secretion of significant quantities of granulocyte-macrophage colony stimulating factor (GM-CSF). The tumors also secrete significant quantities of nerve growth factor (NGF). This observation suggests that taxl may stimulate NGF production and may provide important new insights into the mechanism of HTLV-I induced neurologic disease (tropical spastic paraparesis; also a controversial suggestion of multiple sclerosis).

DNA VIRUS STUDIES

Cytomegalovirus (CMV)

The transforming role of three different CMV strains was studied to determine the gene sequences essential for transformation. The morphological transforming region II (mtr II) of CMV Towne, a strain of CMV, has been localized to a 980-base-pair fragment containing three putative open reading frames (ORFs) of 79, 83, and 34 amino acids. The 2.2 kb colinear region in strain AD169 was also transforming, but the colinear mtrII region in the Tanaka strain had significantly less transforming potential. Analysis revealed the presence of the 79-amino acid ORF in strains Towne and AD169 but not in Tanaka. These comparative data indicate an important role for the 79-amino acid ORF in transformation by CMV.

JC Virus

The JC virus (JCV) has been postulated to be involved in the formation of glial tumors in patients with progressive multifocal leukoencephalopathy. In transgenic mice incorporating the JC virus, the animals develop adrenal neuroblastomas. A cell line derived from neural crest cells has been found that is permissive for JCV. An interesting aspect of these studies has been the finding that the addition of retinoic acid, which stimulates neuronal cell differentiation, elevates the levels of JCV DNA replication and transcription.

Papillomaviruses

Genetic analyses have been carried out to define the open reading frames that encode each of the three E2 proteins of bovine papillomavirus (BPV). These

studies have shown that the amino terminal 200 amino acids (which are highly conserved among the E2 proteins) are necessary for the transactivator function. The transcriptional transactivator functions through direct binding to DNA sequences, and this DNA binding activity is found in the carboxy terminal 100 amino acids.

Continuing studies on transgenic mice harboring the complete BPV-1 genome have revealed differences in the genetic pattern in the tumors they develop. The benign fibroblastic proliferation (fibromatosis) that the mice develop are often aneuploid but without specific abnormalities. This may be a useful model for studying the genetics of papillomavirus-induced tumors.

Simian Virus 40 (SV40)

The transforming region of SV40 contains coding sequences for three proteins, large T-antigen, small t-antigen, and simian virus 40 early leader protein (SELP). Analysis of SV40 T- and t-antigens suggested both proteins have domains that are similar to regions of the adenovirus E1A protein that plays a key role in regulation of transcription. It was demonstrated that t-antigen acts as a trans-activating protein, capable of inducing transcription from promoters that are responsive to E1A.

Human Herpesvirus-6 (HHV-6)

HHV-6 may be synergistic with HIV-1. A number of cell lines consisting of T- and B-lymphocytes as well as cells of other origins, e.g., megakaryocytes, can be infected by HHV-6. Many of these same cell lines are also susceptible to infection by HIV-1. The dual infection of a susceptible cell line by HIV-1 and HHV-6 results in an accelerated loss of viable cells. While these observations are consistent with possible synergistic activity, the actual mechanisms involved are unknown. HHV-6 could contribute directly by stimulating HIV-1 expression or by interacting with HIV-1 long terminal repeat segments affecting virus expression.

Dual infection of CD4+ cells (fresh cells or cell lines) with HHV-6 and HIV-1 or HIV-2 showed enhanced killing and a significant increase in HIV-1 reverse transcriptase (RT) activity. Transactivation of HIV LTRs suggests that HHV-6 may contribute directly or indirectly to the depletion of CD4+ T-cells in AIDS.

In addition, HHV-6 trans-activates HIV-2 and simian immunodeficiency virus (SIV) LTR, but not HTLV-I LTR. Increased HIV-1 LTR trans-activation is obtained in HHV-6-infected cells coinfected with HIV-1 or cotransfected with the HIV-1 tat gene.

GROWTH FACTOR AND ONCOGENE STUDIES

Platelet-derived Growth Factor-Beta (PDGF-ß)

A retrovirus containing the entire human PDGF-ß gene was constructed in order to investigate the in vivo biological activity of its encoded growth factor. When this virus was introduced into newborn mice, it reproducibly generated fibrosarcomas at the site of inoculation. Evidence that the normal growth

factor-coding sequence was unaltered derived from RNAase protection studies and immunoprecipitation analysis. Tumors were polyclonal but demonstrated clonal subpopulations. Moreover, tumor-derived cell lines rapidly became monoclonal upon growth in culture. These findings argue that PDGF-ß overexpression can provide the initiating event for development of a clonal malignancy.

Keratinocyte Growth Factor (KGF)

A novel growth factor specific for epithelial cells was identified in conditioned medium of a human embryonic lung fibroblast cell line. The factor, provisionally termed keratinocyte growth factor because of its predominant activity on this cell type, was purified to homogeneity. KGF was both acid and heat labile and consisted of a single polypeptide chain of approximately 28 kilodaltons. Purified KGF was a potent mitogen for epithelial cells, capable of stimulating DNA synthesis in quiescent BALB/MK epidermal keratinocytes. Lack of mitogenic activity on either fibroblasts or endothelial cells indicated that KGF possessed a target cell specificity distinct from any previously characterized growth factor. Microsequencing revealed an amino-terminal sequence containing no significant homology to any known protein.

Epidermal Growth Factor (EGF)

Alterations affecting the EGF transforming growth factor α (TGF-α)-responsive mitogenic pathway are frequently detected in malignancies. In particular, the EGF receptor (EGFR) molecule has been found overexpressed in a number of human tumors, and TGF-α is produced by a large array of tumor cells. Analysis of human tumor cell lines revealed a strong correlation between expression of TGF α and overexpression of EGFR. Moreover, high levels of EGF-independent tyrosine phosphorylation of the EGFR were detected both in NIH 3T3 cells expressing TGF-α and in high EGFR and TGF-α coexpressing human tumor cell lines. Thus, the two events instituting the EGFR/TGF-α autocrine loop responsible for transformation in vitro may play a role in the development of some human malignancies.

erbB-2

Membrane protein levels of erbB-2 in human mammary cancer were determined in primary and metastatic lesions. Among 57 patients, erbB-2 gene amplification was detected in 11 tumors (19%). In 10 of these patients in whom expression levels could be assayed gene amplification was associated with a high level of erbB-2 protein. Data suggest that overexpression of erbB proto-oncogenes can develop early in breast cancer and be maintained during tumor progression. Comparison of erbB-2 overexpression with clinical disease parameters revealed a correlation of this alteration with inflammatory mammary carcinoma, implying an association of elevated erbB-2 protein levels with enhanced malignancy of the tumor cell in vivo.

Human Gene Map

The human gene map was extended using physical mapping methods to study genes which influence human tumorigenesis. The combination of two gene mapping technologies, somatic cell hybrid analysis and in situ hybridization, has been

used to map 35 distinct human structural genes. The rodent x human hybrid panels were developed using isozyme markers and high resolution G-trypsin banding from a group of over 1,000 hybrid clones. Southern analysis of hybrid DNA and in situ hybridization of tritiated probes to metaphase chromosomes have resulted in chromosomal localization of proto-oncogenes, and genes for growth factors, growth factor and retrovirus receptors, endogenous retroviral segments, retroviral integration sites, and members of the immunoglobulin gene super family.

CHEMICAL AND PHYSICAL CARCINOGENESIS

A central problem in chemical and physical carcinogenesis research is the extrapolation of data from experimental animals to the human population, and within this heterogeneous population, extrapolation among individuals. Epidemiologic and clinical observations provide clues for generating hypotheses. In many cases, clinical investigations and studies using animal models can be used to test these hypotheses, while in other cases, in vitro models that use human tissues and cells collected at the time of autopsy and surgery are more suitable.

Remarkable progress has been made during the last several years in the development of methodology to culture normal human tissues and their epithelial (lining) cells from most major sites of human cancer. The mechanisms of action of carcinogens, tumor promoters, growth factors, and differentiation inducers, can now be investigated at the tissue, cellular, and subcellular levels of biological organization. An integral facet of this strategy is that the same types of tissues and cells from experimental animals can be maintained in identical, controlled in vitro experimental settings so that comparative studies using human and experimental animal material can be conducted. Using in vitro and in vivo models, several research areas are being investigated. These areas include carcinogen metabolism, DNA damage and DNA repair, activation of oncogenes, studies on tumor promotion, and cellular and molecular biology of normal and neoplastic cells. Other areas of active research include multidisciplinary studies of dietary mutagens and carcinogens, and studies on the biochemical epidemiology of human cancer. Highlights for fiscal year 1989 are summarized below.

Model Development for Studying Chemical Carcinogenesis

Remarkable progress has been made during the last few years in establishing conditions for culturing human epithelial tissues and cells. Normal tissues from most of the major human cancer sites can be successfully maintained in culture for periods of weeks to months. Chemically-defined media for long-term culture of human bronchus, colon, esophagus, liver and pancreatic duct have been developed. Primary cell cultures of human epithelial outgrowths have been obtained from many different types of human tissues. Defined culture conditions for normal human epithelial cells from the bronchus, esophagus, pleural mesothelioma and liver have now been established. For example, methods have also been developed to culture pleural mesothelial cells obtained from non-cancerous donors. Pure cultures are initiated by pelleting the mesothelial cells from pleural effusion fluid and inoculating the resuspended cells into FN/C/BSA-coated dishes containing LHC basal nutrient medium supplemented with growth factors. Using this protocol, mesothelial

cell cultures have been established from more than 200 donors. The cells can be subcultured at clonal density with a colony-forming efficiency of more than 10% and high density cultures can be subcultured four to six times before senescence.

These culture systems are now sufficiently established to permit 1) pathobiologic investigations of normal human bronchial epithelial (NHBE) cells, e.g., regulation of growth and differentiation pathways and their dysregulation during carcinogenesis; 2) short- and long-term asbestos carcinogenesis investigations of mesothelial cells; and 3) studies of putative synergistic effects of hepatitis B virus and chemical carcinogens in hepatocellular carcinogenesis.

Newly established cell lines of mouse epidermal keratinocytes in serum-free media were characterized. These lines, even at advanced passage levels, retain epithelial morphology and markers, respond to the induction of terminal differentiation by calcium and by serum, and remain non-tumorigenic. It was found that increased amounts of bovine serum albumin in the medium support cell growth in the absence of bovine pituitary extract; therefore, a new chemically defined medium (LEP/MK4) was formulated and used for further studies on cell differentiation and transformation, currently under way. The BALB/3T3 clone A31-1-1, previously extensively characterized and found susceptible to transformation by a broad spectrum of soluble organic and inorganic carcinogens, was used for pilot studies on penetration, toxicity and transformation by particulate and fibrous materials, including various forms of crystalline silica and asbestos fibers.

Experimental hepatocarcinogenesis in the rat has been used as a model to study the cellular and molecular events during neoplastic development. The research is currently focused on defining the possible role of a stem cell compartment in the liver during oncogenesis as well as in the normal liver. It has been shown that facultative stem cell compartment exists in the liver and these stem cells become a major source of new hepatocytes when the normal regenerative response of the liver is impaired. The liver-derived stem cells are multipotential and are capable of differentiating in vivo into hepatocytes, bile and intestinal epithelia as well as into pancreatic cell lineages. Data obtained strongly indicate that transforming growth factor beta-1 (TGF-ß1) is a key determinant in differentiating the stem cells along the hepatocytic lineage in vivo as well as in vitro. However, during hepatocarcinogenesis, TGF-ß1 may function as an endogenous promoter of the neoplastic process due to its strong growth inhibitory effects on normal hepatocytes in combination with a growth stimulatory effect on transformed liver cells. Moreover, the major source of TGF-ß1 in the liver during later stages of tumorigenesis are the stromal cells and consequently the tumor promoting effect of TGF-ß1 may occur via a paracrine mechanism.

A determining role for metabolic activation/detoxication in susceptibility to transplacental carcinogenesis has been definitively confirmed for a polycyclic aromatic hydrocarbon in a pharmacogenetic mouse model. Detailed mechanistic analysis of the phenomena underlying this relationship is now being studied, including measurement of the enzymatic activity of and gene expression for the relevant Phase I and Phase II enzymes, Western blot of protein levels, and assessment of DNA adducts by the ^{32}P-postlabeling method. A comparison of the induction kinetics in B6D2F$_1$ and D2B6F$_1$ fetuses from responsive and

nonresponsive mothers, respectively, has shown that similar levels of AHH activity are attained following transplacental injection of MC regardless of the phenotype of the mother. However, fetuses from nonresponsive D2 mothers maintained their induced P-450IA1 levels, as measured enzymatically, for at least 48 hr, whereas values for fetuses from responsive mothers had declined to control levels by this time. A gene for an important Phase II enzyme, uridine diphosphoglucuronic acid transferase, has been found to be well expressed in fetal mouse liver. When this multifactorial analysis is eventually completed, it will provide a depiction of genetics-related modulation of tumorigenesis of unprecedented completeness.

The interaction of two important categories of human-exposure chemicals, ethanol and N-nitrosamines, is being studied in mice and patas monkeys. In mice, oral coadministration of ethanol with three different nitrosamines has resulted in significant increases in tumors initiated by the nitrosamine in three target organs. It has been postulated that this effect of ethanol is due to competitive blockage of nitrosamine metabolism in liver, leading to greater delivery of dose to sensitive targets. In confirmation of this hypothesis, coadministration of ethanol with N-nitrosodimethylamine resulted in a striking prolongation of the circulating half-life of the carcinogen. An even more dramatic retardation of clearance was seen in the patas monkey, suggesting that the findings may be extrapolatable to the human. The further hypothesis that the increase in circulating levels might result in a greater number of tumor initiating events was supported by the finding that ethanol cotreatment led to a 3- to 9-fold increase in amounts of promutagenic O^6-methylguanine in lung DNA. Further analysis of these models will continue to elucidate not only the specific interactions between nitrosamine and ethanol, but also the more general task of evaluating, in terms of risk assessment, the net outcome of multifactor exposure of humans.

Cytochrome P-450s and Metabolism of Carcinogens

The cytochrome P-450s are a superfamily of enzymes which metabolize a wide array of compounds including xenobiotics such as drugs and carcinogens, and endobiotics such as steroids and prostaglandins. In animals, multiple forms of these enzymes are expressed simultaneously either constitutively or after administration of inducers. To define the contribution of a given cytochrome P-450 to the metabolism of specific drugs and carcinogens, it is important to express these enzymes individually in cells which lack endogenous background activity for these enzymes. Toward this goal expression systems in which individual cytochrome P-450s are synthesized from their coding DNA sequences are being developed. Success in this effort will enable the identification of putative human mutagens and carcinogens as well as to define the contribution of each of these enzymes to toxicity, mutagenesis, and cell transformation by chemical carcinogens. To achieve this goal, infectious recombinant vaccinia viruses and infectious recombinant retroviruses containing the full-length coding cDNA sequences for mouse cytochrome P-450IA1 and P-450IA2 have been constructed. Human and rodent cells infected with the recombinant viruses expressed high levels of the authentic size proteins that were enzymatically active and displayed substrate specificities diagnostic of the respective enzymes. Employing the recombinant cytochromes it has been demonstrated that the cytochrome P-450IA2 selectively activates heterocyclic arylamines and cytochrome P-450IA1 preferentially activates aromatic hydrocarbons; this

preferential selectivity is mutually exclusive at limiting substrate concentrations.

Another approach to the identification and quantitation of P-450 isozyme species in tissues and organs is the use of epitope-specific monoclonal antibodies (MAbs) to P-450 isozymes. Eight libraries of MAbs to different forms of P-450 (LM2, LM4, MC-B, PB-B, PCN-E, ETOH, SCUP, and RLM5) were established and phenotyping of animal and human tissues was carried out by radioimmunoassay (RIA), reaction-phenotyping and immunohistochemistry. 3-Methylcholanthrene (MC)-inducible P-450s were found in the lung, kidney, liver, and intestine of C57/BL mice and rats which were treated with MC. The MC forms of P-450 were also found in human placenta and lymphocytes of smoking women. However, the level of growth hormone- and male sex-dependent P-450 RLM5 was not greatly affected by MC induction. Ethanol-inducible P-450j was found in human adult livers and pregnenolone 16 α-carbonitrile-inducible P-450 PCN-E was found in both adult and fetal human livers. Application of crude coal tar to skin of neonatal rats predominantly induced P-450 MC-B in both epidermis and liver but the P-450 PB-B form was also induced in liver. Treatment of adult male rats with chlotrimazole and diphenylhydantoin induced phenobarbital PCN and ethanol inducible-forms, respectively, in liver. These results indicate that MAbs are useful probes for identification of P-450s which are induced by the administration of various drugs and chemical carcinogens.

Polyclonal (PAb) and/or monoclonal antibodies (MAb) have been raised against peptide sequences that are unique for a given P-450 enzyme, while the MAbs are generated against purified, native P-450 enzymes. The production of antipeptide PAb presents the opportunity to generate antibodies in a more rational and timely manner. In addition, the creation of P-450-specific anti-peptide PAb results in a reagent that is not only useful in numerous immunoassays but can also be used in the purification of the native molecule, which in turn can be used for production of MAb. The current research efforts center around the generation of specific anti-peptide PAb against sequences unique to either P-450d or P-450c. These two rat P-450 enzymes, induced following exposure to 3-methylcholanthrene, are key enzymes in the metabolism of carcinogens of the aromatic amine and polyaromatic hydrocarbon classes, respectively. While several unique regions have been identified in both enzymes, the initial emphasis is on the creation of antisera against peptide sequences unique to P-450d, as there is no available antibody which specifically recognizes P-450d. The anti-peptide PAbs are examined for use as reagents in ELISA and western blot analysis, and for inhibition of enzyme activity.

A large number of P-450s have been purified from rodent tissues and their substrate specificities examined by in vitro reconstitution assays. Few P-450s have been purified from man, however, owing to the paucity of readily available human tissues. Purification of these enzymes from human tissue is further complicated by the large degree of genetic variability among individuals and the difficulties in obtaining homogeneous preparations of specific P-450 forms. A cDNA cloning and expression approach to the study of human P-450s has been applied. cDNA probes and antibodies to rodent P-450s are being used to screen λgt11 expression libraries constructed from different human liver and lung RNA preparations. The cDNAs are being sequenced and then used to produce active enzymes in cell culture. The enzymes are examined for

their abilities to carry out the oxidation of common therapeutically used drugs and to activate carcinogens and mutagens. cDNAs have been isolated and several human P-450s have been expressed including IA1, IA2, IIA3, IIB1, IIB2, IIC8, IIC9, IID1, IIE1, IIF1, IIIA4, IIIA5, IIIA6 and IVB1. The chromosomal locations of the genes in each P-450 subfamily have been determined. In addition, scientists have cloned, sequenced, and expressed human microsomal xenobiotic epoxide hydrolase and NADPH-P-450 oxidoreductase. cDNA probes to human P-450s and antibodies against rodent P-450s were used to probe individual human liver specimens for P-450 gene expression to locate livers that do not express a particular P-450. These samples are further examined for the existence of mutant human genes.

To determine the mechanism by which P-450 genes are transcriptionally activated by inducing agents, genomic clones for two rat clofibrate-inducible genes designated IVA1 and IVA2 were isolated from a lambda EMBL3 library. These genes are both transcriptionally activated by hypolipidemic agents. A highly conserved 19 base pair region was identified just upstream of the start sites for these genes. This region may be involved in regulation of the IVA1 and IVA2 genes. Interestingly, the latter gene is constitutively expressed in kidney.

DNA Damage and Repair

Although DNA repair has been studied extensively in human fibroblasts, lymphoid cells, and neoplastic cells, little information is available concerning DNA repair in normal human epithelial cells. Using cultured human bronchial epithelial and fibroblastic cells, studies have been initiated to investigate DNA damage and repair caused by chemical and physical carcinogens as examined by alkaline elution methodology, BND cellulose chromatography, unscheduled DNA synthesis, and high pressure liquid chromatographic analysis of the formation and removal of carcinogen-DNA adducts. Human bronchial epithelial cells have been found to repair single-strand breaks in DNA damaged by X-irradiation, ultraviolet (UV)-radiation, chromate, polynuclear aromatic hydrocarbons, formaldehyde, or N-nitrosamines at rates similar to bronchial fibroblasts.

Normal adult human tissues and cultured bronchial epithelial cells and fibroblasts exhibit O^6-alkylguanine-DNA alkyltransferase activity in vitro by catalyzing the repair of the promutagenic alkylation lesion O^6-methylguanine from DNA. Alkyltransferase activity varies in the different human tissues tested in the decreasing order of liver, colon, esophagus, peripheral lung and brain. Formaldehyde inhibits repair of O^6-methylguanine and potentiates the mutagenicity of an alkylating agent, N-methyl-N-nitrosourea, in normal human fibroblasts. In some experimental studies, repeated exposure to alkylating agents has led to an increase in O^6-methylguanine-DNA alkyltransferase activity, i.e., an adaptive response. It was shown that human bronchial epithelial cells do not adapt and increase their DNA repair capability. This finding has important implications in carcinogenesis caused by low doses of N-nitrosamines. The effects of cigarette smoke condensate (CSC), catechol and smoke "conditioned" media on the activity of O^6-methylguanine-DNA alkyltransferase (O^6-MT) and the effects of uracil-DNA glycosylase (UDG) on cultured human bronchial epithelial cells, HuT-292 cells and BEAS-2B cells, is currently under investigation. The activity of these two DNA repair enzymes is also being measured in the alveolar macrophages and peripheral blood

lymphocytes of smokers and nonsmokers. Inter- and intra-individual variation in these activities is up to 100-fold and sixfold, respectively, O^6-MT activity is lower in macrophages of smokers which is consistent with the hypothesis that tobacco smoke inhibits repair of alkyl-DNA adducts.

Molecular, cellular, and clinical abnormalities in patients with xeroderma pigmentosum (XP), with the dysplastic nevus syndrome (DNS) of familial cutaneous melanoma, and with Bloom's syndrome (BS) are being studied. New assays utilizing plasmids as tools to measure DNA repair, ligation and mutagenesis at the molecular level in cultured human cells have been developed. Utilizing a shuttle vector plasmid, pZ189, it was shown that there is a restricted spectrum of mutations induced in UV-treated plasmid replicating in XP cells of complementation groups A and D. DNS cells introduced more mutations into UV-treated pZ189 than normal cells but had fewer tandem mutations. The major UV photo-product, the T-T cyclobutane dimer, was found to be only weakly mutagenic in XP, DNS and normal lines. It was found that photoproduct frequency was not the major determinant of UV mutation frequency in plasmids replicated in human cells. Utilizing a linearized replicating plasmid, scientists demonstrated a reduced ability of BS cells to ligate plasmids in vivo and that this ligation process was error prone. The BS cells also introduced more deletion mutations than normal cells into the recircularized plasmids. Oxidative DNA damage to pZ189 produced by treatment with Cu(II) plus hydrogen peroxide produced site-specific damage at polyguanosines. A Registry of XP patients has been established. A 3-year clinical trial of cancer chemoprevention demonstrated that a high dose (2 mg/kg/da) of 13-cis retinoic acid (Accutane), administered orally, is effective in preventing formation of new skin cancers in patients with XP.

Transforming Growth Factor-beta (TGF-beta)

Growth factors have been shown to play a pivotal role in molecular and cellular biology. Indeed, the current excitement in research about one such growth factor, TGF-beta comes from its multiple actions on almost every type of cell and its potential for therapeutic use in common clinical conditions for which there are presently no adequate pharmacological agents. Although TGF-beta was originally identified in an assay which measured its ability to enhance the growth of fibroblasts ("transformation"), its true importance is as a mediator of normal cellular physiology, in particular during normal formation of tissues (as in embryogenesis), as well as during their response to injury (as in inflammation and repair). Almost all cells have been shown to make TGF-beta in one of its molecular forms, and essentially all normal cells have receptors for TGF-beta.

TGF-beta is a highly stable peptide, consisting of two identical chains, each of 112 amino acids. It was first isolated and characterized definitively from human platelets and placentas, as well as bovine kidneys, and has also been cloned. This molecule is now known as TGF-beta 1, and the universality of its action is emphasized by the fact that its amino acid sequence is identical in man, monkey, cow, pig, and chicken. Four additional closely related types of TGF-beta have subsequently been identified and their separate genes cloned. The cloning of these five new genes will allow for large-scale production of these peptides by recombinant DNA technology; this has already occurred with TGF-beta 1.

TGF-beta has many actions. It can stimulate proliferation in some cells, especially in connective tissue, while it is a potent inhibitor of proliferation in others, such as lymphocytes and most normal epithelial cells. Moreover, it can regulate other processes which have little to do with cell division, such as the synthesis of collagen and other critical molecules of the extracellular matrix of bone, cartilage, and connective tissue. There is now an extensive literature on the role of TGF-beta during inflammation and repair. Platelets are the most concentrated source of TGF-beta in the body, and TGF-beta is released from them at sites of tissue injury. This process starts a cascade of events leading to tissue repair. The effect of TGF-beta on collagen synthesis is especially noteworthy, since it has been shown that TGF-beta enhances gene transcription for this molecule, which has such a critical role in providing the structural strength of healing wounds, as well as serving as an essential part of the matrix of bone and cartilage.

These actions are of obvious therapeutic relevance for potential clinical applications, including surgical wound healing in debilitated patients or those undergoing chemotherapy, treatment of diabetic, decubitus, and varicose ulcers, and treatment of burns. Animal studies have shown that TGF-beta can enhance wound healing in many experiments, but human use has not yet begun. An important new application has been the intra-ocular use of TGF-beta to promote retinal reattachment to the underlying tissue after retinal detachment, a common cause of human blindness. This has been achieved experimentally in rabbits and is awaiting further development for clinical use. There are also disease states in which excessive production of TGF-beta, or abnormal sensitivity of target cells to its actions, may contribute to the pathogenesis of fibrosis, as has been suggested from human and animal studies on pulmonary fibrosis, hepatic cirrhosis, scleroderma, keloids, and rheumatoid arthritis.

Oncogenes in Chemical Carcinogenesis

The identification of specific transforming genes (oncogenes) in both human and experimental tumors has stimulated great interest and intensive efforts in many laboratories to clarify the roles of those genes in the pathogenesis of cancer. As many of the known oncogenes derive from normal elements of the mammalian genome, there is a real possibility that the biochemical mechanisms of neoplastic transformation may be definable by thorough analysis of the properties of the oncogenes and their cellular homologs. Of special interest in chemical carcinogenesis are oncogenes, such as the *ras* family of genes, that behave as dominant genetic elements and that are activated to this behavior by a mutation in a single base of DNA. This mechanism of activation can be caused, in principle, by mutagenic chemical carcinogens, which can be provisionally identified as such by their genotoxic effects. The mechanism of action of such agents, long considered to involve damage to DNA, may eventually be reconciled with molecular virology, and the crucial events in cellular transformation, by both chemical and biological agents, defined through analysis of the activation and behavior of oncogenes. Detection and critical evaluation of the roles of dominant oncogenes, especially mutant genes of the *ras* and *erbB* families was a major area of research in chemical carcinogenesis during the past year.

Last year the *neu* oncogene, an oncogene present in chemically induced brain tumors in rats, was described. During this year, it has been demonstrated

that neu is consistently activated in diverse species, with both hamster and mouse schwannomas containing transforming sequences associated with this oncogene. This oncogene is the only growth-factor receptor-protein kinase type oncogene known to be mutationally activated in an experimental tumor, the rat schwannoma.

The expression of the ras oncogene protein product, p21, was studied in pathology specimens of rats, mice and humans. Several different monoclonal or polyclonal antibodies were used to demonstrate that immunohistochemical reactivity depended on the tissue fixative, tissue and specific antibody. Some antibodies reacted with epitopes in p21 or other proteins in the cytoplasm of cells within normal tissues of all three species. Cell membrane immunoreactivity could only be found on tumor cells or transformed in vitro cell lines. A well-characterized anti-21 monoclonal antibody was found to react to a series of proteins in normal and transformed cells but never with cell membranes. Several control methods for proving specificity of the antibody-antigen reaction were developed.

Urinary bladder tumors induced by a nitrosamine in rats were found to express high levels of ras p21. Transfection of NIH 3T3 cells with tumor DNA produced transformed colonies for only 2/11 tumors. After tumor DNA amplification by the polymerase chain reaction (PCR), still only 2/22 tumor DNAs were found to have mutations at codon 12 of H-ras. More than 60% (12/20) tumors were found to have mutations in codon 61 of H-ras by restriction fragment length polymorphism after PCR. Thus, for the first time it was found, by immunohistochemistry, that increased expression of ras p21 was associated with a high rate of ras mutations in a chemically-induced rodent tumor.

A new method for detecting oncogene and retroviral proteins in fixed tissue sections was developed. In vitro cell lines with specific retroviral infections (e.g., HIV-IIIB, and SIV-MnIV) or activated or amplified ras or erbB-2 oncogenes were used to produce cell pellets. The pellets were used to prepare sections for immunohistochemical reactivity with monoclonal and polyclonal antibodies to oncogene or retroviral proteins. The use of cells with known expression of viruses or oncogenes proved extremely valuable in accessing protein expression in autopsy specimens of rodents and humans.

An amplification of the c-myc, N-myc or L-myc gene and overexpression of the c-raf sequence are common features of small cell lung carcinomas. Thus, the major objective of an ongoing project is to determine the role of these genes in lung carcinogenesis by overexpressing c-raf and/or c-myc genes in human lung cells, and reversion of the malignant phenotype by plasmids capable of anti-sense RNA transcription.

Retroviral recombinants pZip-raf and pZip-myc were constructed to examine the role of the c-raf-1 and c-myc proto-oncogene in lung carcinogenesis. Immortalized human bronchial epithelial cells (BEAS-2B) transfected with pZip-raf DNA and pZip-myc DNA gave rise to undifferentiated carcinomas (raf/myc tumors) when tested in athymic nude mice, whereas c-myc or c-raf transfected cells are non-tumorigenic. The raf/myc tumors expressed markers of small cell lung carcinomas, i.e., neuron-specific enolase and neurosecretory granules. In addition, BEAS-2B cells transformed with the c-raf and c-myc proto-oncogenes, and derived tumor cell lines acquired HLA class II antigen expression.

The c-raf-1 gene has been identified as the predominant transforming gene of three radiation-resistant head and neck cancer cell lines in the NIH 3T3 transfection assay (SQ-20B, JSQ-3, SCC-35). NIH 3T3 cells transformed with SQ-20B DNA also became radiation-resistant, suggesting a correlation between the presence of c-raf sequences and the radiation-resistant phenotype. Inhibition of the c-raf function by introduction of anti-sense raf transcribing plasmids into the SQ-20B cell line reverted not only the tumorigenic phenotype but also reduced the radiation resistance. As a consequence of these experiments, the construction of an inducible promoter system for anti-sense sequences in human cells was undertaken.

Five human bronchial epithelial cell lines have been established from NHBE cells by SV40 early region gene transfer. One additional cell line has been established following transfection of SV40 early region genes into bronchial epithelial cells known to contain an abnormality of the short arm of chromosome 11 from an individual who was cancer-free; the resulting cell line is tumorigenic in athymic nude mice. At least one other cell line with a chromosome 3p deletion has developed weak tumorigenicity. Several different mutant ras oncogenes have resulted in malignant transformation. These cell lines, and tumor cell lines established from the nude mouse tumors, are being utilized to study aspects of multistage carcinogenesis, including chromosomal changes, progressive changes in response to inducers of squamous differentiation and the development of invasiveness.

Tumor Promotion Studies

Studies are underway to aid in understanding the early events in the interaction of phorbol ester tumor promoters with cells and tissues. Particular attention is being devoted to the analysis of the major phorbol ester receptor, protein kinase C. The bryostatins, macrocytic lactones, activate protein kinase C and compete for phorbol ester binding. They only induce a subset of the typical phorbol ester responses, however. The biochemical mechanisms for these differences are being explored through immunoblotting of protein kinase C, comparison of phosphorylation patterns, tritiated bryostatin binding analysis, and comparison of effects on cloned and chromatographically separated protein kinase C isozymes. Structure-activity analysis suggests that bryostatin derivatives differ in the degree to which they are bryostatin-like in their actions rather than phorbol ester-like. Computer modeling indicates excellent fit to the previously derived phorbol ester pharmacophore and is consistent with the structure-activity relations. Other structural classes of protein kinase C modulators under investigation include 7-beta-meprounic acid 3-p-hydroxy-benzoate, cyclic diglycerides, and phorbol derivatives modified in the tigliane ring structure or its functional groups. A second research direction is to identify the targets of irritant but non-promoting phorbol-related derivatives. It has been shown that resiniferatoxin acts as a selective ultrapotent capsaicin analog. Typical phorbol esters exert their inflammatory activity through both resiniferatoxin-sensitive and insensitive pathways. The latter correlate better with promoting activity. Current efforts are directed at identification and characterization of resiniferatoxin receptors.

Rodent renal tumor promoters including barbital sodium and nitrilotriacetic acid were used to develop an in vitro rodent and human cellular assay system to study mechanisms of renal tumor promotion. Rat and human renal epithelial

cells were cultivated in vitro and effects on colony formation, colony growth and cell-cell communication by the lucifer yellow dye transfer technique using the microinjection technique were studied. Two rat renal cell lines, NRK and NK-4, expressed differing levels of gap junctions and cell-cell communication. Despite this difference in cell surface physiology, the two cell lines responded similarly to the growth stimulating effects of the two tumor promoters. Attempts at immortalization of the human renal cells with SV40 T antigen proved difficult. Although cells appeared to survive for long periods of time, establishment of a permanent cell line was slow. Cell-cell comunication in the human cells was variable between colonies. Tumor promoters had no obvious effect on cell survival or growth.

The pathogenesis of hepatic and renal tumors induced by nongenotoxic carcinogens or promoted by tumor promoters were studied in rats and mice. Levels of DNA synthesis in target hepatocytes or renal tubular cells were evaluated by Brdu immunohistochemistry or tritiated thymidine autoradiography.

While almost all renal tumor promoters are associated with increased levels of DNA synthesis (hyperplasia) in target cells for promotion in the renal tubules, some renal toxins, e.g., di(2-ethylhexyl)phthalate (DEHP), cause a marked hyperplasia without evidence of carcinogenesis or tumor promotion after initiation by N-nitrosoethylurea. Liver tumor promoters, however, often caused a transient increase in levels of hepatocyte DNA synthesis, while rarely causing chronic persistent increases.

Food-Derived Mutagens and Cancer

During the last decade, a number of studies have shown that the cooking of meat, under certain circumstances, produces mutagens which may contribute to a person's risk for cancer of the stomach, large intestine, or other organs. Of these mutagens, the most important are a group known as the aminoimidazoazaarenes (AIAs), also referred to as heterocyclic amines. Their importance derives from the fact that human exposure to them is highest, since they are formed at relatively low household cooking temperatures by grilling, frying and broiling meats. Although a number of related heterocyclic amine carcinogens can be formed by heating amino acids and foods, they are not produced under normal household cooking conditions (200 to 300 degrees Centigrade).

A number of AIAs have been purified from cooked ground beef, a major protein source in the western diet. All AIAs characterized to date, with one exception, 2-amino-1-methyl-6 phenylimidazo[4,5-f]pyridine (PhIP), are very potent mutagens in a bacterial assay system known as the Ames test. PhIP is a relatively weak mutagen, but it is present in tenfold greater concentrations in cooked beef than any of the other AIAs, and is the most potent AIA in mutagenicity studies utilizing mammalian cells rather than bacteria.

Thus far only three of the AIAs, referred to as IQ (2-amino-3-methylimidazo[4,5-f]quinoline), MeIQ (2-amino-3-methylimidazo[4,5-f]quinoline), and MeIQx (2-amino-3-methylimidazo[4,5-f]quinoxaline), have been evaluated in long-term rodent bioassays, and all three have been found to induce a variety of tumors including tumors of the liver, forestomach, and colon. Synthesis of N-OH-IQ, and N-acetoxy-IQ and IQ-N-sulfate, the reactive metabolites of IQ, also has been accomplished. Synthesis and characterization

of the major DNA-IQ adducts and examination of DNA-IQ adducts in rodents and nonhuman primates is underway and the role of specific cytochrome P-450s in the metabolic activation of IQ is being evaluated. One IQ adduct, the C8 guanine adduct, was synthesized and shown to be formed in vitro from either N-OH-IQ or N-acetoxy-IQ reacting with DNA. Several DNA-IQ adducts, including the C8-guanine-IQ, were found in the livers of cynomolgus monkeys fed IQ. DNA-IQ adducts were also detected in kidney, colon, stomach and bladder, and these adducts were identical in all organs examined. Recently, three cynomolgus monkeys receiving daily oral doses of IQ at 20 mg/kg were diagnosed with hepatocellular carcinoma. The latent period for tumor induction (30 months) is similar to the latent period for liver tumor induction by diethylnitrosamine, the most effective hepatocarcinogen in nonhuman primates. Recent studies have been conducted on the metabolism of some of the AIAs in nonhuman primates. Studies have been examining the disposition of IQ in monkeys and identifying urinary and fecal metabolites of IQ which may be indicative of pathways of carcinogen activation or detoxification. Following administration of ^{14}C-IQ (2 μmol/kg) to cynomolgus monkeys by nasal-gastric intubation, blood levels declined rapidly from 1 to 8 hr following dosing. This was followed by a slow decline from 8 to 72 hr. Approximately 30-45% of the dose was excreted in the urine by 8 hr and by 72 hr 45-60% had been excreted. Through HPLC analysis it was observed that IQ is extensively metabolized in monkeys to at least 4 urinary metabolites. No IQ itself was found in the urine. One of the metabolites found was IQ-N-sulfamate. In addition, two glucuronides of IQ were tentatively identified by their sensitivity to ß-glucuronidase. Structural identification of these metabolites is currently underway.

Only a few studies have been performed thus far in humans, but all have demonstrated that mutagens and their metabolites are present in the urine of individuals eating fried meat. The specific chemical responsible for the mutagenicity in these early studies were not identified. However, recent developments in both chemical detection, as well as detection using antibodies against the AIAs, have allowed detection of specific AIAs in humans after consumption of fried meat. In addition, during the past year it was shown that a single type of cytochrome P-450 is responsible for adding a chemical residue to most if not all of the AIAs. Studies both in humans and animals have shown that this type of P-450 exhibits a high degree of individual variation and that its levels are increased by environmental pollutants such as cigarette smoke. This is of fundamental importance, since both risk and individual susceptibility to the mutagenic effects of AIAs may be directly related to the activity of this particular type of cytochrome P-450. Therefore, any risk estimation for an individual must consider both the level of exposure and how the individual metabolizes a particular chemical. By employing currently available methods for measurement of parent AIAs, their metabolites, and DNA adducts, it should be possible to obtain indices of both individual exposure and the response to that exposure.

Biochemical Epidemiology

The primary goal of biochemical epidemiology is to identify individuals at high cancer risk by obtaining pathobiological evidence of high exposure of target cells to carcinogens and/or increased host susceptibility due to inherited or acquired factors. Laboratory methods have been developed recently to be used in combination with analytical epidemiology to identify

individuals at high cancer risk. These methods include techniques to assess specific host susceptibility factors; assays that detect carcinogens in human tissues, cells, and fluids; cellular assays to measure pathobiological evidence of exposure to carcinogens; and methods to measure early biochemical and molecular responses to carcinogens.

Currently available techniques exist that would allow the utilization of biochemical and molecular measures to better characterize exposure to carcinogens, to serve as intermediate endpoints on the path to malignancy, to identify measures which halt or reverse this process, and to investigate the mechanisms of human carcinogenesis. Included in these investigations would be the following: (1) efforts to evaluate body burden of chemical carcinogens in studies of occupational and general environmental cancer risk factors; (2) sophisticated analyses of air, water, and biological specimens for carcinogenic and mutagenic substances in conjunction with specific analytical studies; (3) search for evidence of viral inflection including viral segments or oncogenes in the DNA of individuals at high risk of cancers that may be associated with infectious agents or heritable states; (4) evaluation of disturbances in immune function as they may relate to malignancies, particularly those of the hematopoietic system; (5) investigation of the relationship between micronutrients and a variety of epithelial cancers; and (6) determination of the relationship between macronutrients, including dietary fat and subsequent hormonal changes, to subsequent risk of breast, endometrial, and perhaps colon cancer.

Classical epidemiology and xenobiochemical studies have revealed questions relating to the genotoxic effects of environmental contaminants in humans. Development of assays for carcinogen exposure at the molecular level in humans has therefore been a major focus of research and from the battery of assays that are currently available, both immunological and physico-chemical approaches are being further developed. Since each type of assay system clearly has its own advantages and disadvantages, the use of more than one corroborative assay has been of importance. The problems that appear to confront the development of assay systems for human biomonitoring are essentially twofold: the levels of material present in macromolecular complexes challenge the detection limits of conventional assay systems, and complex mixtures of adducted materials confound simple assay systems. Following recognition and definition of cross-reactivity profiles for antibodies raised against aromatic-DNA adducts, protocols that combine immunoaffinity chromatography, high performance liquid chromatography (HPLC), fluorescence spectroscopy, gas chromatograph/mass spectroscopy and 32P-postlabeling are being developed. The development of HPLC-32P-postlabeling techniques for the measurement of either hydroxylated residues in DNA (for example, 8-hydroxydeoxyguanosine) or alkylated residues in DNA (for example, ^{06}methyl- or N7-methyldeoxyguanosine) are also in progress. With regard to oxidative damage, 8-hydroxydeoxyguanosine in particular, electrochemical detection is being explored.

EPIDEMIOLOGY AND BIOSTATISTICS

Case-control and cohort studies designed to evaluate hypotheses in cancer etiology continued to receive emphasis. Case-control studies of selected cancers were undertaken when 1) high-risk communities were identified, 2) key

hypotheses were testable, or 3) special resources became available. Laboratory analyses were incorporated into studies when they might clarify exposures and other risk factors, preclinical responses, or mechanisms of carcinogenesis. Special emphasis was given to studies aimed at understanding the high rates of certain cancers in blacks and other minority groups.

Also undertaken were descriptive surveys to identify trends in cancer incidence and mortality, as well as groups having unusual cancer patterns. An updated atlas, which illustrates geographic patterns of cancer mortality in the nonwhite populations by state economic area in the 1970s, is nearing completion. Preliminary findings indicate the emergence of elevated mortality rates for prostate cancer along the South Atlantic coast, but there is a general trend toward geographic uniformity over time for most other individual cancers, as seen for whites in the atlas completed last year.

Diet, Nutrition, and Cancer

Evidence from international correlations and migrant populations suggests that diet and nutrition are important in cancer etiology, but specific dietary risk factors have not been well established in case-control and cohort studies. The Program continues to test and generate hypotheses on the role of diet and nutrition on cancer risk.

The risk of colorectal cancer has been shown to be correlated with higher levels of mutagens in the feces of certain populations eating a typical Western diet. Although the specific fecal mutagens have not yet been identified, the fecapentaene mutagens were thought to pose a risk on the basis of their genotoxicity and widespread presence in stool samples from North Americans. However, results from a case-control study of fecal mutagens and colorectal cancer showed a decrease in fecapentaene excretion among the cases, as compared to controls, which could not be explained by the effects of diagnostic work-up or surgery. The non-fecapentaene *Salmonella* TA-98 mutagens were significantly elevated in cases, stimulating investigations as to the dietary origins of this mutagenicity.

In a large community-based case-control study of cervical cancer in the U.S., women in the highest quartiles of intake of carotenoids, vitamin A, vitamin C, and folacin had adjusted relative risks of invasive squamous-cell cervical cancer comparable to women in the lowest quartiles, although their micronutrient intake was estimated to be 3-4 times as high. Risk was not affected by the intake of various food groups, including fruits and vegetables. These generally negative findings stand in contrast to previous epidemiologic studies, suggesting a protective role for micronutrients.

In a multicenter study in four geographic areas of the U.S., fruit consumption was shown to be protective for oral cancer, with risks among those in the highest quartile being only half those in the lowest. The association held for fruits both high and low in vitamin C, suggesting that other nutritive or non-nutritive components may be involved. Low fruit intake was also shown to increase the risk of esophageal cancer in the high-risk area along coastal South Carolina.

In a study at the University of California at San Francisco, the risk of breast cancer is being investigated in relation to constituents of breast

fluid secretions and to dietary factors among American women. The greatest amount of breast fluid was collected from women 35-50 years of age, those with early onset of menses and ones who had lactated, while the lowest yields were from women of Asian ancestry. The breast fluid varied from colorless through black, though the color was not related to known breast cancer risk factors. Proliferative breast disease was 8.5 times as likely to occur in women with cholesterol beta-epoxide in their breast fluid than in women without detectable levels of the metabolite.

A study at Harvard University found that the consumption of lactose-rich foods, the dietary source of galactose, increased the risk of ovarian cancer. The activity of galactose transferase, involved in the conversion of galactose to glucose, was also found to be significantly lower in ovarian cancer cases than in controls. This finding provides a new etiologic clue to the origins of ovarian cancer, about which little is known.

Investigators at the University of Hawaii observed that the risk of prostatic cancer rose as the area of muscle in the arm increased in men of Japanese ancestry. They did not find any association between the area of fat in the arm and prostatic cancer, indicating that lean tissue rather than body fat may play a role in the development of this cancer.

Nutritional hypotheses were examined by several collaborative studies in areas of the world with exceptionally high rates of certain cancers. In a case-control study in Shandong, China, stomach cancer patients tended to consume more salted foods and sour pancakes and less fresh vegetables. Protective effects were most prominent for vegetables of the allium class (e.g., garlic), which is of interest since experimental studies have found allium to have tumor-inhibitory properties. Laboratory analyses of the sour pancakes and allium vegetables are underway. In a multicenter study of stomach cancer in high- and low-risk areas of Italy, garlic consumption was shown to lower risk, as was the intake of fresh (but not cooked or preserved) fruits and vegetables.

In Linxian, China, consumption during adulthood of pickled vegetables was not found to be a risk factor for esophageal cancer, contrary to previous suspicions. Cases had lower fluid and higher wheat and corn intake, similar to Iran where exceptionally high esophageal cancer rates also occur. Large-scale randomized nutrition intervention trials are continuing in Linxian to evaluate the preventive effects of various vitamins and minerals.

Infectious Agents

Advances in laboratory techniques, such as in viral isolation and detection, provide new approaches for investigating the role of viruses in cancer etiology. Employing DNA hybridization techniques to assess the association between type-specific human papillomavirus (HPV) infection and cervical cancer risk, a case-control study in Latin America found HPV types 16 or 18 associated with a fivefold excess risk. In a case-control study of cervical dysplasia in Washington, D.C., a fourfold excess risk was associated with the detection of any HPV type. Blacks were more likely than whites to have HPV detected, consistent with higher incidence rates for cervical cancer among blacks. Another study of dysplasia in Washington, D.C. found a tenfold risk associated with detection of any HPV type. After adjustments for other known

risk factors, the elevated risks for cervical dysplasia or cancer persisted in all three studies.

NCI studies on the relationship of adult T-cell leukemia (ATL) to T-cell lymphotropic virus type I (HTLV-I) have focused on Jamaica, where rates of this viral-associated leukemia are high. By coupling HTLV-I exposure data to a population-based leukemia registry, the risk for ATL in seropositive cases was found to be elevated 35-fold compared to controls. Also, modeling ATL incidence with HTLV-I prevalence suggested that risk for ATL is especially high among individuals exposed early in life to the virus, with a 3-5% lifetime risk for leukemia. Studies of special populations have identified that about 20% of infants of HTLV-I seropositive mothers become infected, primarily due to breast feeding.

Sexual transmission of HTLV-I has been evaluated in several populations, confirming prior observations that male-to-female transmission predominates, accounting for the disproportionate excess of female positives in older age groups. Female-to-male transmission may involve cofactors, such as the presence of other sexually transmitted diseases and ulcerative genital lesions. Parenteral transmission via transfusion and through sharing of needles is also linked to the spread of HTLV-I. Finally, recent evidence suggests that coinfection with human immunodeficiency virus (HIV) and HTLV-I may accelerate progression to acquired immunodeficiency syndrome (AIDS) compared to individuals infected with HIV alone.

In a study at Johns Hopkins University, sera and peripheral blood lymphocytes from HIV-infected individuals were studied 18, 12, and 6 months prior to the first positive Western blot and at the time of seroconversion. Polymerase chain reaction (PCR) amplification data showed that all seroconverters were PCR positive at the time of seroconversion, and most were PCR positive 6 to 12 months before the first positive Western blot.

Tobacco and Alcohol

Several NCI investigations have further clarified the role of tobacco and alcohol in cancer risk. In the largest investigation of oral and pharyngeal cancer yet conducted (1200 cases and 1300 controls), smoking and drinking were shown to be the dominant risk factors. The large study size enabled the first clear demonstration of the independent effect of alcohol consumption, as well as an analysis showing that smoking and drinking tend to combine more in a multiplicative than additive fashion to cause oral cancer. Among heavy smokers and drinkers, the risks of oral/pharyngeal cancer rose over 35-fold. Risks fell sharply following cessation of smoking, suggesting that it affects primarily a late stage in oral carcinogenesis.

Tobacco and alcohol were found to be the main determinants of esophageal cancer in coastal South Carolina, where mortality rates for this tumor have long been elevated among blacks. Consumption of local moonshine whiskeys, reported by nearly 90% of the black male cases, appeared at least partly responsible for the high rates in this area. Laboratory analyses are underway to identify possible carcinogenic ingredients of moonshine whiskeys.

Occupational/Environmental Carcinogens

Most chemicals known to be carcinogenic in humans were first identified in studies of occupational groups, whose exposures are often heavier and of longer duration than those typically encountered by the general population. Case-control and cohort studies are underway to investigate a wide range of exposures, including acrylonitrile, formaldehyde, pesticides, organic and inorganic dusts, metal fumes, and organic solvents (e.g., trichloroethylene, perchloroethylene, methylene chloride, benzene, benzidine).

In a case-control study conducted in Kansas, the risk of soft-tissue sarcomas was found to be elevated among farmers reporting the use of animal insecticides. Risks rose to nearly fivefold among those first exposed in the 1940s, and appeared to be more strongly linked to organochlorine insecticides than other chemicals. In studies of U.S. Department of Agriculture employees, leukemia was elevated among agricultural extension agents, and non-Hodgkin's lymphoma among soil and forest conservationists. For both tumors, risks rose with the number of years employed. To further assess risks from pesticide exposures among farmers, analyses are underway of data from case-control studies of lymphatic and hematopoietic cancers in Nebraska, Iowa, and Minnesota.

Detailed analyses of occupational data collected during the National Bladder Cancer Study indicated that the proportion of bladder cancer attributable to occupation was about 20% in men and 5% in women. The relative risks were generally similar in men and women, although the frequency of exposure to occupational carcinogens was substantially lower in women. While the contribution of high-risk occupations to bladder cancer among nonwhite men was similar to that among white men, there appeared to be racial differences in their specific exposures, even among workers in the same industry or job title category.

Under an NCI/NIOSH Interagency Agreement, a case-control study of lung cancer in the Teamsters Union revealed that long-term truck drivers, who were potentially exposed to diesel exhaust, have about a 50-90% increased risk after control for smoking. Industrial hygiene studies in the industry are underway to clarify the extent of actual levels of diesel exposure among truck drivers. These findings are interesting in view of earlier NCI studies linking bladder cancer risk, especially among truck drivers, to occupational exposures to motor vehicle exhausts.

In a collaborative study in Shanghai, China, the risk of lung cancer was increased among women reporting greater 1) high temperature wok cooking, 2) house smokiness and eye irritation when cooking, and 3) use of rapeseed cooking oils. This association is intriguing since Chinese investigators have reported that rapeseed volatiles are mutagenic in the Ames test, and since it might contribute to the high incidence of lung cancer (notably adenocarcinoma) among nonsmoking Chinese women. In Shenyang, China, the risk of lung cancer rose with greater exposure to indoor pollution from coal-burning Kang and other home-heating devices that generate high levels of polycyclic hydrocarbons. Industrial sources of pollution were also implicated in this study, with a threefold increased risk of lung cancer among males living within one kilometer of a large copper smelter that emits inorganic arsenic and other metallic pollutants.

Genetic Susceptibility

Based on epidemiologic and clinical observations by NCI investigators, plus collaborative studies with laboratory scientists, family cancer syndromes have provided clues to mechanisms of host susceptibility. Recent developments include localization of the gene for the more common form of neurofibromatosis (NF-1) to the long arm of chromosome 17, near the gene for the receptor of the nerve growth factor, and the less common form (NF-2, acoustic neuromas) to the long arm of chromosome 22.

NCI epidemiologists reported a decade ago that dysplastic nevi were precursor lesions for melanoma in members of melanoma-prone families. Approximately 10% of all melanomas occur in individuals with a family history of melanoma. In these families, melanoma and dysplastic nevi represent an autosomal dominant single-gene trait. In collaboration with laboratory investigators, the susceptibility gene has been localized on chromosome 1p36.

Extramural scientists have investigated oncogenes and tumor suppressor genes in a number of modelling studies. The two classes of genes have been incorporated into stochastic models of carcinogenesis and applied to epidemiologic data from studies of Wilms' tumor and cancers of the colon and breast.

Studies of pharmacogenetics have revealed a relationship between lung cancer susceptibility and the extent to which the antihypertensive drug debrisoquine is metabolized. The risks were more pronounced for cell types of lung cancer, such as squamous and oat-cell carcinomas, that are most strongly linked to smoking. Differences in the ability to metabolize debrisoquine have been found between blacks and whites. A NCI case-control study of lung cancer has been initiated to follow up on these findings.

Radiation

A study of adult leukemia and lymphoma, utilizing prepaid health plans, indicated that diagnostic x-rays may not be causally related to these diseases, but simply associated with conditions that portend their development. For multiple myeloma, however, there was a suggestion of increasing risk with increasing number of x-rays. Continuing emphasis is being given to case-control studies of lung cancer to clarify the effects of indoor radon exposure in homes. A new survey is underway to evaluate cancer incidence and mortality, with special emphasis on childhood leukemia, among U.S. populations residing near nuclear reactor facilities.

Ultraviolet (UV) radiation has been investigated as a cause of melanoma and non-melanoma skin cancer. For melanoma, childhood and intermittent (recreational) exposures were found to be especially important, while for other skin cancers, cumulative (occupational) exposures play a key role. Also clarified were predisposing host factors in the form of skin complexion for all types of skin cancer, and dysplastic nevi for melanoma. Since sunlight is the major source of UV radiation, there has been concern about the depletion of stratospheric ozone, especially in view of recent reports of "ozone holes" over Antarctica and decreasing trends in stratospheric ozone levels (mostly during winter months in the northern hemisphere). However, surface measurements of solar UV radiation have shown no increasing trend as yet, but

further monitoring of UV exposures and skin cancer incidence is warranted in collaboration with other Federal agencies.

Medications

In a collaborative case-control study of 23,000 Swedish women who used menopausal hormones, an excess risk of endometrial cancer (showing a dose-response with both duration of use and strength of medication) was noted for estrogens unopposed by progestational agents. For women who used only the combination regimen (i.e., estrogen plus progestogen), no excess risk of endometrial cancer was detected. However, for those women who switched from unopposed to opposed regimens, some excess risk persisted. Evaluation of breast cancer risk revealed a 60% increase in breast cancer after 10 or more years of replacement estrogen therapy. This excess was not diminished by the addition of progestogen to the regimen, and in fact, the risks were somewhat higher and seen with shorter durations of use.

ACTIVITIES IN THE OFFICE OF THE DIRECTOR

The Division of Cancer Etiology is responsible for planning and directing a national program of basic research including laboratory and epidemiologic studies on the causes and natural history of cancer, on the molecular biology, natural history, and transmission of HIV, and on the development of a vaccine for AIDS. Basic research on methods and approaches to cancer prevention is also within the Division's sphere of activities. These research efforts are carried out in the intramural laboratories and branches of the Division as well as extramurally, utilizing research grants, cooperative agreements, interagency agreements and contracts. The Office of the Director coordinates, plans, and directs a program of national and international research in cancer etiology and also serves as a focal point for the Federal Government for the synthesis and dissemination of clinical, epidemiological and experimental data related to cancer etiology and cancer prevention.

Activities in the area of environmental carcinogenesis are located in the Office of the Director. A number of cooperative projects and collaborations with other Federal agencies have been carried out under interagency agreements with the U.S. Environmental Protection Agency (EPA) and the National Institute for Occupational Safety and Health (NIOSH). In addition to managing and serving as Project Officers on these interagency agreements, staff from the Office of the Director interface with State agencies, industrial and trade organizations, academic institutions and professional societies, serving a primary role in dissemination of information on environmental problems and industrial exposures in carcinogenesis.

The Interagency Collaborative Group on Environmental Carcinogenesis (ICGEC), organized within the Office of the Director 16 years ago, also serves as a vehicle for information exchange. The ICGEC was originally constituted to provide a mechanism for interagency contacts to secure access to data bases; it has provided, indirectly, a stimulus for development of projects in the area of environmental and occupational carcinogenesis. It consists of representatives from 28 agencies or subagencies, and meets every few months. By October 1989 there will have been 90 meetings. Topics of meetings held this year are "Radon Exposure" and "Smokeless Tobacco."

Staff in the Office of the Director participate on the Task Force on Environmental Cancer and Heart and Lung Disease, for which EPA is the lead agency. This Task Force was formed some years ago in response to Congressional stimulation as a result of mandates under the Clean Air Act. A Congressionally mandated DHHS report entitled "Research Activities of Relevance to the Clean Air Act: Biennial Report to Congress" is prepared by the Office of the Director every 2 years.

The Registry of Experimental Cancers is directed and maintained by staff of the Office of the Director. The objectives of the Registry of Experimental Cancers are the storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents) and the use of such information for research and educational purposes. The Registry has acquired a total of 8,762 (1,425 since last year) single or group accessions from investigators outside the NCI, and approximately 70,166 records have been coded. Thirty-nine investigators have come to the Registry

for study and consultation on single or multiple visits. The Director General of the World Health Organization (WHO) designated the Registry of Experimental Cancers as the WHO Collaborating Center for Reference on Tumors of Laboratory Animals on October 26, 1976, and the Pan American Health Organization (PAHO) renewed this collaboration on July 19, 1983. This is the only such repository in the world to be so designated by the WHO. The Registry facilitates communication between U.S. scientists and those of other countries, now numbering 153, which are members of WHO.

The Office of the Director supports, by staff and by contractor, the NCI Chemical Selection Working Group (CSWG) for NCI nominations of chemicals to the National Toxicology Program (NTP). It also maintains a Chemical Selection Planning Group (CSPG) which works with a contractor to develop nominations and make decisions on chemicals to be submitted to the CSWG. Another information dissemination activity involves preparation, under contract, of the PHS-149 "Survey of Compounds Which Have Been Tested for Carcinogenic Activity." Previous contracts provided for the preparation of volumes for 1974-75, 1976-77, and 1979-80, which have been distributed. The present contract, with Technical Resources, Inc., provides for the preparation of volumes for 1989-91. The 1987-88 volume of the survey was compiled and is being printed by the Government Printing Office; it will be placed on sale through the Superintendant of Documents In addition, DCE will distribute copies to over 600 regulatory and public health agencies and research institutes throughout the world. The latest volume contains entries on 748 chemicals extracted from 781 articles selected from 655 journals published during 1987 to 1988. Also distributed were new cumulative indices containing the chemical names, Chemical Abstract Service (CAS) registry numbers and accession numbers for all chemicals included in PHS-149 up to and including 1988. Work is progressing on the completion of the 1989-1990 volume which will be published in 1991. Screening efforts of the 1991 literature is progressing on schedule.

International Agency for Research on Cancer (IARC) Monograph Series "Evaluation of the Carcinogenic Risk of Chemicals to Humans"

The Division supports a Cooperative Agreement with IARC which is managed by staff of the Office of the Director. IARC is located in Lyon, France, and the title of the project is "Evaluation of Carcinogenic Risks to Humans." IARC established this program in 1970 and monographs have been published in volumes so entitled for a large number of chemicals. Thus far 47 volumes have been published and several are in production; the volumes contain monographs in which the carcinogenic risk to man of chemicals, groups of chemicals and, more recently, of industrial and occupational exposures, as well as life-style factors, is evaluated on the basis of results in experimental animals, studies in in vitro systems and epidemiologic studies. The monographs also contain background information on the chemicals under consideration such as chemical and physical properties, analysis, occurrence, production, use, and estimated human exposures from all sources. This information is provided to IARC by NCI through a resource contract currently held by Technical Resources, Inc. The IARC monographs have become a highly respected and authoritative reference source for countries around the world. Another IARC activity supported under this agreement is the compilation of a listing of laboratories around the world into a compendium entitled "Survey of Chemicals Being Tested for Carcinogenicity." The IARC initiated this survey in 1973 on a worldwide

basis; thus far 13 surveys have been published and the fourteenth survey is in preparation. These surveys are made available so that laboratories involved in carcinogenesis research can coordinate their testing and research, thus avoiding unnecessary duplication.

Registry of Tumors in Lower Animals

The Division continues to support the Registry of Tumors in Lower Animals (RTLA) which is located at the Smithsonian Institution in Washington, DC. The RTLA is the focal point through which information on neoplasms in lower animals is channelled and maintained. Neoplasms and tumor-bearing animals of invertebrate or cold-blooded vertebrate species are collected, studied, classified, and preserved at the Registry, which maintains the largest collection of lower animals in the world. In addition to maintaining a specimen depository, the RTLA provides a diagnostic service to biologists in many fields and consequently assists in the identification of clusters of neoplasms in feral animals that may have been exposed to environmental carcinogens in their habitat. Another ongoing activity of the RTLA is the collection and indexing of all scientific literature pertinent to neoplasia in lower animals, including experimentally induced, genetically influenced, and "spontaneous" tumors. Together with a computerized listing of the Registry's specimen accessions, this constitutes virtually all the information available on neoplasms in lower animals.

Special Projects on Environmental Carcinogenesis

Centers for Disease Control (CDC): Studies on the Human Health Consequences of Polybrominated Biphenyl (PBB) Contamination of Farms in Michigan

The cohort of individuals developed to monitor the effects of the 1973 animal feed error has now reached 4,038. The increase of slightly over 100 individuals is due to the enrollment of babies born to cohort mothers, others who previously declined to participate and members of the Mt. Sinai Hospital cohort. In aggregate, these more than compensate for the <1% annual loss due to death and "dropouts." Contractual expert support is in place and working to create a master file that will permit the expeditious manipulation of the data in the files containing the information accumulated since the project's initiation. Internal coordination has been strengthened within the Michigan Department of Public Health between the Center for Environmental Studies (in which this project resides), the State Register, and the Office of Management and Information Systems.

Environmental Protection Agency (EPA): Performance of Collaborative Studies in the Area of Environmental Cancer

No studies were funded during this reporting period under this interagency agreement. However, dialog between NCI and EPA staff continued to identify projects of interest to both agencies and suitable for support under this mechanism.

National Institute for Occupational Safety and Health (NIOSH): Conduct of Research on Occupational Carcinogenesis

Interaction between the two agencies' staffs have been frequent and productive during the past year and seven projects are in various stages ranging from near completion to preliminary discussions. The large and complex epidemiological study "Occupational Cancers in Workers Exposed to Silica and Asbestos in the North Carolina Dusty Trades Industries" is in a one-year no-cost extension. "Industry and Occupational Coding of Death Certificates" has continued with the cooperation of the State governments but the National Center for Health Statistics has suggested that projected short-falls in funding this year and next may jeopardize its continuance. The "Industry-Wide Acrylonitrile Study," with its NIOSH industrial hygiene component, is continuing. It is anticipated that it will be completed in FY 1990 without further funding under this agreement. The three new projects for this year are underway. Work on "Use of Multiple Causes of Death Data for the U.S., 1968-1985" has begun under contract. The contract for "Feasibility Assessments for New Topics" is being finalized by NIOSH. Approval has been given to study 2,4-D as the first issue and approval will be sought to subsequently study the compounds Alachlor and 2-Biphenylphenol. The steering committee has met on the "International Workshop on Retrospective Exposure Assessment in Occupational Epidemiology" and the activity is scheduled to take place in March 1990. Early discussions have resulted in the decision that staff members of both agencies, supplemented by a small group of other experts, will meet in October 1989 to hold a workshop on "Biochemical Monitoring in Epidemiology." A joint staff meeting will be held at the end of FY 89 to develop concepts for additional projects for the future.

Microbiological Associates Inc.: In Vitro Evaluation of Chemical Candidates for In Vivo Testing -- Mouse Lymphoma Assay and Salmonella Typhimurium Assay

The contractor is completing year two of the present three-year contract. The assays completed have supported the chemical nomination process to the National Toxicology Program, complemented the Division's investigations of several compounds, and continued to supply data to the Chemical Carcinogenesis Research Information System (CCRIS) data base of the National Library of Medicine's TOXNET. A manuscript on the mutagenicity of six compounds abused as recreational drugs has been accepted for publication and data is being collected for the preparation of several other manuscripts. Preliminary steps have been completed that are required for the initiation of the competitive renewal of these two assay contracts. That recompetition will commence in July 1989.

Technical Resources, Inc.: A Resource to Support the Chemical, Economic, and Biological Information Needs of the Division of Cancer Etiology (DCE) and to Provide Chemical Process, Production and Economic Information to the International Agency for Research on Cancer (IARC)

During this reporting period, the contractor prepared summary sheets on 15 chemicals which were considered for nomination for carcinogenicity testing by the National Toxicology Program (NTP) at three meetings of the Chemical Selection Working Group. In addition, information data sheets were prepared on 5 candidate chemicals for consideration by the Chemical Selection Planning Group. NCI, as in the past, continues to be the primary source for

nominations of candidate chemicals to the NTP. The contractor also provided support for three IARC working group meetings. Data for Sections 1 and 2 (Chemical and Physical Data and Production, Use, Occurrence and Analysis) were prepared for a total of 19 monographs. A contractor representative attended all meetings and, in addition, chaired the Chemistry Subgroup at two of these meetings. The Chemical Carcinogenesis Research Information System Data Base (CCRIS) was maintained and updated and now contains entries on 688 unique chemicals in the carcinogenicity file, on 985 chemicals in the mutagenicity file and on 94 chemicals in the tumor promotion file. A tumor inhibitor file has been added to CCRIS during this reporting period. Data are being entered into the maintenance file which is expected to become available to the public within the next 12 months. CCRIS continues to reside in the National Library of Medicine's TOXNET system. Periodic tape updates were provided to the National Technical Information Service for use by commercial data services. The Bioassay Report Summary Handbook was updated by the addition of summaries on 30 NTP Technical Reports.

Chemical Carcinogenesis in Nonhuman Primates

Staff of the Office of the Director direct a large project on chemical carcinogenesis in nonhuman primates. This project continues to evaluate the potential carcinogenic effects of a multitude of substances in nonhuman primates. These include nitroso- compounds, antineoplastic, and immunosuppressive agents, "classical" rodent carcinogens, food additives, food components, and environmental contaminants. Over the past year the major findings were on the heterocyclic amines most recently under evaluation in the colony, i.e., 2-amino-3-methylimidazo[4,5f]quinoline (IQ) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). Of a group of 20 monkeys which have received 20 mg/kg of IQ since 1985, three are dead with primary hepatocellular carcinoma (HCC) (one metastatic to lungs) and four have biopsy-proven HCC. One monkey receiving 10 mg/kg has died with metastic HCC. Futhermore, studies have been initiated which examine DNA IQ-adducts in peripheral blood cells. Typical IQ-adducts have been detected in blood cells from several of the monkeys receiving IQ. Dosing with MeIQx (10 mg/kg was started 6 to 9 months ago). Thus far, none of the ten animals receiving the compounds have shown signs of tumor development. The other test compound which has produced tumors for the first time is melphalan. The two melphalan monkeys involved had the same type of poorly differentiated to undifferentiated sarcomas in the perineal and perivaginal regions. Further studies on cell lines derived from these tumors are being done to elucidate the role of melphalan in this tumor development.

OFFICE OF THE DIRECTOR

CONTRACTS ACTIVE DURING FY 88

Institution/Principal Investigator/ Contract Number	Title
Centers for Disease Control Rebecca Schilling Y02-CO-70529	Studies on the Human Health Consequences of Polybrominated Biphenyl (PBB) Contamination of Farms in Michigan
Environmental Protection Agency (EPA) W. Farland and F. Ulvedal Y01-CP-80205	Performance of Collaborative Studies in the Area of Environmental Cancer
Microbiological Associates Inc. John Harbelle Richard San N01-CP-71084	In Vitro Evaluation of Chemical Candidates for In Vivo Testing -- Mouse Lymphoma Assay and Salmonella Typhimurium Assay
National Institute for Occupational Safety and Health (NIOSH) Roy M. Fleming Y01-CP-60505	Conduct of Research on Occupational Carcinogenesis
Technical Resources, Inc. Beverly Campbell N01-CP-71114	Survey of Compounds Which Have Been Tested for Carcinogenic Activity
Technical Resources, Inc. Harry Seifried N01-CP-71082	Resource to Support the Chemical, Economic, and Biological Information Needs of the Division of Cancer Etiology (DCE) and to Provide Chemical Process, Production and Economic Information as Support to the International Agency for Research on Cancer
Smithsonian Institution John Harshbarger N01-CP-51031	Operation of a Registry of Tumors in Lower Animals
Hazleton Laboratories America, Inc. Dan W. Dalgard N01-CP-51013	Induction, Biological Markers and Therapy of Tumors in Primates

International Agency for
Research on Cancer
Antero Aitio
5-U01-33193-05

IARC Monographs on the Evaluation
of Carcinogenic Risks to Humans

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP03509-26 OD

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Carcinogenesis, Chemotherapy and Biological Markers in Nonhuman Primates

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
Others:	S. M. Sieber	Deputy Director	OD, DCE	NCI
	R. H. Adamson	Director	OD, DCE	NCI

COOPERATING UNITS *(if any)*
Hazleton Laboratories America, Inc., Vienna, VA (D. Dalgard)

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.0	1.5	2.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

This project continues to evaluate the potential carcinogenic effects of a multitude of substances in nonhuman primates. These include nitroso-compounds, antineoplastic and immunosuppressive agents, "classical" rodent carcinogens, food additives, food components, and environmental contaminants. Over the past year the major findings were on the heterocyclic amines, most recently introduced into the colony, i.e., 2-amino-3-methyl-3H-imidazo[4,5A]quinoline (IQ) and 2-amino-3,8-dimethyl[4,5-f]quinoxaline (MeIQx). Of a group of 20 monkeys which have received 20 mg/kg of IQ since 1985 three are dead with primary hepatocellular carcinoma (HCC) (one metastatic to lungs) and four have biopsy proven HCC. One monkey receiving 10 mg/kg has died with metastatic HCC. Furthermore, studies have been initiated which examine DNA IQ-adducts in peripheral blood cells. Typical IQ-adducts have been detected in blood cells from several of the monkeys receiving IQ. Dosing with MeIQx (10 mg/kg) was started 6-9 months ago. None of the ten animals receiving the compounds have shown signs of tumor development. The other test compound which has produced tumors for the first time is melphalan. The two melphalan monkeys involved had the same type of poorly differentiated to undifferentiated sarcomas in the perineal and perivaginal regions. Further studies on cell lines derived from these tumors are being done to elucidate the role of melphalan in this tumor development.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. P. Thorgeirsson	Expert	OD, DCE	NCI
S. M. Sieber	Deputy Director	OD, DCE	NCI
R. H. Adamson	Director	OD, DCE	NCI

Objectives:

Due to the phylogenetic and biological similarities to man, nonhuman primates are possibly the most reliable experimental animals for predicting carcinogenic effects on humans. The major objectives at the onset of this project were: 1) to obtain comparative data on the response of nonhuman primates and known rodent carcinogens; 2) to evaluate suspected human carcinogens in food and in the environment; and 3) to evaluate long-term toxic and carcinogenic effects of antineoplastic and immunosuppressive agents. Other objectives include obtaining model tumor systems for testing the effectiveness of antitumor agents, and studies of a variety of factors involved in hepatocarcinogenesis.

Methods Employed:

The present colony, which consists of 470 animals (May 1, 1989) is comprised of four species: Macaca mulatta (rhesus), Macaca fascicularis cynomolgus), Cercopithecus aethiops (African green) and Galago crassicaudatus (bushbabies). Forty-two of the monkeys are adult breeders which supply infants (20-30 per year), who are reared by their mothers and weaned at approximately 4 months of age. The majority of the monkeys are housed in an isolated facility which contains only animals committed to this study.

The administration of test compounds is continued until a tumor is diagnosed, or until a predetermined exposure period is completed. The compounds are administered subcutaneously, intravenously, intraperitoneally, or orally. For oral administration to newborn monkeys, the compound is added to the Similac formula at the time of feeding. When the monkeys are 6 months old, compounds given orally are incorporated into a vitamin mixture which is given on a slice of bread. An alternate way of giving compounds orally is to incorporate them into baited foods or administer by intubation. The antineoplastic and immunosuppressive agents are administered at doses likely to be given in a clinical situation. Environmental contaminants are given at levels 10- to 40-fold higher than the estimated human exposure level. The remainder of the test chemicals are administered at maximally tolerated doses, judged by clinical observations, weight, blood chemistry, and hematology.

Twenty-nine substances are currently, or have been under investigation. These include antineoplastic and immunosuppressive agents (procarbazine, adriamycin, 1-methylnitrosourea (MNU), melphalan, azathioprine and cyclophosphamide), food additives (cyclamate, saccharin, butter yellow); food components (aflatoxin B_1, cycad, sterigmatocystin, IQ, MeIQx); environmental contaminants (DDT, arsenic, cigarette tobacco smoke condensate); "classical" rodent carcinogens (urethane, 3-methyl-cholanthrene, 2-acetylaminofluorene, (2-AAF) copper chelate of N-OH-AAF, dibenzpyrene and dibenzanthracene); nitroso-compounds (dimelthylnitros-amine, diethylnitrosamine, dipropylnitrosamine, 1-nitrosopiperidine, N-methyl-N'-nitro-N-nitrosoguanidine).

Major Findings:

The major findings over the past year are on the carcinogenicity of IQ and melphalan, neither of which have previously been shown to produce tumors in monkeys. In a group of 20 monkeys receiving 20 mg/kg of IQ through nasogastric tube feeding since 1985, seven have developed hepatocellular carcinoma, verified either at autopsy or through punch biopsies. The cumulative IQ dose ranged from 19-26 grams. Alphafetoprotein levels were elevated in the three monkeys that were sacrificed and autopsied. Two of the monkeys had extensive liver involvement, one with metastases to the lungs. One monkey in a group of 20 monkeys receiving 10 mg/kg has developed hepatocellular carcinoma with lung metastases, verified at autopsy. Studies are ongoing on the metabolic activation of IQ, DNA adducts, and repair. In general, the same N-hydroxylated derivatives as in the rat were found in the monkeys. These active metabolites form adducts with DNA. ^{32}P-postlabeling studies showed that the DNA adducts are the same in the rat and the monkey. Interestingly, these DNA adducts can also be detected in peripheral blood cells of monkeys currently receiving IQ. Correlation between DNA adducts in blood cells and tumor development will be made over the next year.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04548-17 OD

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Registry of Experimental Cancers/WHO Collab. Ctr. for Tumors of Lab Animals

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Harold L. Stewart	Scientist Emeritus	OD, DCE	NCI
Others:	Umberto Saffiotti	Acting Head, REC	OD, DCE	NCI
	Bernard Sass	Vetinary Med. Ofcr.	OD, DCE	NCI
	Annabel G. Liebelt	Expert	OD, DCE	NCI
	Eliza Chavez	Tech. Inform. Spec.	OD, DCE	NCI

COOPERATING UNITS *(If any)*

LAB/BRANCH
Office of the Director

SECTION

INSTITUTE AND LOCATION
NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.1	2.1	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The objectives of the Registry of Experimental Cancers are the storage and retrieval of pathological material and data on cancers and other lesions of laboratory animals (primary rodents) and the use of such information for research and educational purposes. During the current year the Registry has acquired 1,425 single or group accessions from investigators outside the NCI reaching a total of 8,762; a total of approximately 70,166 records have been coded. During this year, 39 investigators have come to the Registry for study and consultation on single or multiple visits.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project

Harold L. Stewart	Scientist Emeritus	OD, DCE	NCI
Umberto Saffiotti	Acting Head	OD, DCE	NCI
Bernard Sass	Veterinary Medical Officer	OD, DCE	NCI
Annabel G. Liebelt	Expert	OD, DCE	NCI
Eliza Chavez	Tech. Inform. Specialist	OD, DCE	NCI

Objectives

1) The storage and retrieval of pathologic material and data on cancers and other lesions of laboratory animals (primarily rodents); 2) the use of such pathologic material and data for research and educational purposes.

Methods Employed

The methods employed in the work of the registry involve the selection of protocols, pathologic material, including histologic slides, paraffin blocks, and illustrations in the form of lantern slides, gross photographs, and photomicrographs in black and white and in color. The work of the Registry also includes the collection of records of experiments, reprints and references on this material. The Registry of Experimental Cancers possesses a large collection of spontaneous and induced cancers and other lesions. The pertinent information on the collection is indexed and many of the data have been entered into the computer. The Registry accesses material from investigators at NCI, other institutes of NIH, other Governmental agencies, industrial laboratories, and universities here and abroad. A total of 8,762 (1,425 during the current year) single or group accessions from investigators outside of NCI have been processed. The Registry prepares study sets of slides, with explanatory notes, relating to particular cancers of rodents.

The Registry has study sets of slides on "Comparative Pathology of Hematopoietic and Lymphoreticular Neoplasms;" "Induced and Spontaneous Tumors of Small Intestine and Peritoneum in Mice;" "Neoplastic and Nonneoplastic Lesions of the Lymphoreticular Tissue in Mice;" "Induced Tumors of the Liver in Rats;" "Tumors and Nonneoplastic Proliferative Lesions of the Lungs of Mice;" Mammary Tumors in Mice;" "Pulmonary Metastases in Mice;" "Neoplasms and Other Lesions of Praomys (Mastomys) Natalensis;" "Malignant Schwannomas of Rats;" "Tumors of the Harderian Gland of Mice;" "Induced Tumors of Kidney of Rats;" "Spontaneous Gastric Adenomatosis, Polyps and Diverticula;" "Tumors of Adrenal Gland of the Rat", "Malignant Schwannomas of Mice;" and "Comparative Pathology of Malignant Tumors of the Soft Tissues and a Few Miscellaneous Tumors of Several Animal Species." These study sets, with descriptive material, are loaned to investigators who request them. Four loans have been made this year to countries abroad. Investigators come to the Registry for study and consultation. This year there have been single or multiple consultations with 39 individuals.

The Director General of the World Health Organization (WHO) designated the Registry of Experimental Cancers as the WHO Collaborating Centre for Reference on Tumors of Laboratory Animals on October 26, 1976 and the Pan American Health organization renewed this collaboration in March 1988. This is the only such registry in the world to be so designated by the WHO. The Registry will expand communications between U. S. scientists and those of other countries, now numbering 153, which are members of the WHO.

Major Findings

The functions (outlined in objectives) of the Registry in the field of cancer research are such that there will be no major findings to report.

Publications:

Hayes HM Jr, Sass B. Testicular tumors; species and strain variations. In: Cancer growth and progression. comparative aspects of tumor development Kaiser HE, Ed. Dordrecht, The Netherlands: Kluwer Publishers 1989;5:106-18.

Hoch-Ligeti C, Stewart HL. Cardiac tumors in laboratory rodents-comparative pathology. In: Comparative aspects of tumor development. cancer growth and progression, Kaiser HE, Dordrecht, The Netherlands: Kluwer Academic Publishers, 1989;5:152-75.

Liebelt AG. Malignant neoplasms occurring in organ transplant recipients. In: Etiology of cancer in man. Cancer growth and progression, Kaiser HE, Dordrecht, The Netherlands: Kluwer Academic Publishers, 1989;6:136-67.

Liebelt AG, Sass B, Sobel HJ, Werner RM. Spontaneous nephroblastoma in a strain CE/J mouse. a case report. Toxicol Pathol, 1989;17:57-61.

Rehm S, Ward JM, Liebelt AG. Mixed adenocarcinoma, mammary gland, mouse. In: Jones TC, Mohr U, Hunt RD, eds. Integumentary system and mammary glands. Monographs on pathology of laboratory animals. New York: Springer-Verlag (In Press).

Sass B. Bovine lymphoma - epidemiology, diagnosis, transmission, pathology. Comparative aspects of tumor development, Cancer growth and progression, In: Kaiser, HE, Dordrecht, The Netherlands, Kluwer Academic Publishers, 1989;5:202-10.

Sass B. Etiology, morphology and pathogenesis of proliferative an hyperplastic lesions and neoplasms of mouse mammary gland. In: Kaiser HE, Ed. Dordrecht, The Netherlands: Cancer growth and progression. Comparative aspects of tumor development, Kluwer Publishers, 1989;5:122-31

Sass B, Hayes, H. Chemodectomas of man and animals. In: Progressive stages of malignant neoplastic growth, Kaiser HE, (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05551-02 OD

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Liposomes as Carriers for Anti-HIV Agents

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. J. Parker	Expert	OD, DCE	NCI
Others:	S. M. Sieber	Deputy Director	OD, DCE	NCI
	J. N. Weinstein	Sr. Investigator	LMB, DCB	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Liposomes are under investigation as carriers of 2′,3′-dideoxynucleotides (ddNT's) in an effort to enhance antiviral activity of these compounds in monocytes-macrophages (M/M) infected with the human immunodeficiency virus type-1 (HIV-1). Cells of the M/M lineage are known to play key roles in the dissemination of the virus and pathogenesis of acquired immunodeficiency syndrome (AIDS). The antiviral effects of 2′,3′-dideoxycytidine (ddC), 2′,23′-dideoxycytidine-5′-triphosphate (ddCTP) and liposome-entrapped ddCTP [L(ddCTP)] were compared in M/M cells infected with HIV-1. These treatments inhibited virus replication at nanomolar drug levels in the order ddC > ddCTP = L(ddCTP). Studies on drug stability and uptake showed that a large portion of ddCTP dephosphorylated before entering cells, whereas L(ddCTP) remained stable over days and was probably taken up by endocytosis. The response to L(ddCTP) suggests that the capabilities of liposomes for targeting drugs to macrophages in vivo can potentially be exploited for improving the therapeutic index of dideoxynucleotide drugs.

Project Description

Names, Title, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. J. Parker	Expert	OD, DCE	NCI
S. M. Sieber	Deputy Director	OD, DCE	NCI
J. N. Weinstein	Sr. Investigator	LMB, DCB	NCI

Objectives:

The major goal of this project is to study the effects of liposome entrapment of ddNT's in an effort to enhance their activity against HIV-1. Since liposomes are degraded by phagocytic uptake into macrophages, they may provide a means to direct entrapped anti-viral agents to macrophages which are one of the cell types known to harbor HIV-1. Furthermore, liposome entrapment has been shown to afford entrapped materials a modest degree of protection against degradation of circulating enzymes and other serum components. These characteristics of liposomes may be of particular importance since ddNT's are not readily taken up by cells and are extensively degraded in the presence of serum.

Major Findings:

Stability of radiolabelled ddCTP in the presence of culture medium (D-MEM/ 10% FCS) containing M/M cells indicated a rapid decomposition to the corresponding monophosphate (70% in 1 hr) followed by further dephosphorylation to a compound which co-eluted with ddC. After a 72 hr incubation, over 90% of the radiolabel co-eluted with ddC.

Studies on the cellular uptake of ddC and ddCTP in M/M cells showed a much higher rate for ddC than for ddCTP. The rate of ddCTP uptake was found to be similar to the rate at which ddCTP is dephosphorylated, suggesting that ddCTP mainly enters cells in the nucleoside form. Liposomes released less than 10% of their entrapped ddCTP within 4 days in the presence of cells and culture medium at 37°C. In contrast the corresponding nucleoside ddC was released much more rapidly from liposomes than was ddCTP.

At 62.5 nM ddCTP, L(ddCTP) and L(H2O)+ddCTP were equally potent in inhibiting p24 expression, producing 90-95% suppression of viral growth over a 3-week period. Control liposomes not containing ddNT's did not decrease viral replication at any of the lipid concentrations tested. In contrast ddC was effective in inhibiting p24 expression (91%) at concentrations as low as 15.6 nM. These findings suggested that ddC is 4-5 times more active in supressing viral growth than either ddCTP or L(ddCTP).

Methods Employed:

1. Identification and quantitation of ddCTP and its metabolites in media and serum was achieved by a combination of reverse-phase and ion exchange HPLC using both UV and radioactivity detection. Samples containing liposome entrapped ddNT's or ddNS's were treated with Triton-X prior to HPLC analysis in order to release the entrapped material.

2. ddCTP was entrapped in liposomes composed of phosphatidylserine/lecithin /cholesterol (molar ratio 18:37:45) by extrusion under high pressure at efficiencies of 8-10%. Leakage of liposome entrapped compounds was monitored in stirred dialysis cells at 4° or 37°C in the presence or absence of serum.

3. Peripheral blood monocytes were obtained from leukapheresed healthy HIV-1 antibody-negative donors and purified by elutriation to yield 99% monocyte cultures. Cultured cells were infected with an HIV-1 isolate recovered and propogated in M/M cells.

4. Infected M/M cells were placed in tissue culture wells and treated with free or liposome entrapped compounds on days 1, 3, 5, and 7 following infection. On days 3, 5, 7, 10 and 12 after infection, samples of supernatant from each well were tested for the presence of p24 viral-core protein by ELISA.

Publications:

1. Szebeni J, Wahl SM, Wahl LM, Gartner S, Popovic M, Parker RJ, Black C, Weinstein JN. Inhibition of HIV in monocyte/macrophage culture by 2',3'-dideoxycytidine-5'-triphosphate, free in liposomes. AIDS Res Hum Retroviruses (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05576-02 OD

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Expression of ras and Collagenase in Primary Tumors vs. Metastases

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
Others:	M. Ballin	Visiting Fellow	OD, DCE	NCI
	A. R. Mackay	Visiting Fellow	OD, DCE	NCI
	J. R. Hartzler	Biological Lab. Worker	OD, DCE	NCI
	C. C. Sinha	Biologist	OD, DCE	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.75	0.25

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In this study we examined whether tumor progression and metastatic development is associated with amplification and/or increased expression of the ras oncogene. It was also examined whether type IV collagenolytic activity corresponds with metastatic capacity, both of which have been shown to be coordinately induced through ras transfection. The results from two tumor models indicate that H-ras amplification is not required for development of metastases, and that high p21 levels do not lead to increased metastatic efficiency. Two major metalloproteinases of 67 and 92 kDa were expressed by N-nitrosomethylurea (NMU)-induced mammary tumors and the ras transfectants. Type IV collagen degrading metalloproteinase activity was higher in the metastatic tumors than the normal mammary glands, but did not differ between primary tumors and metastases, or between metastatic and nonmetastatic tumors.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. P. Thorgeirsson	Expert	OD, DCE	NCI
M. Ballin	Visiting Fellow	OD, DCE	NCI
A. R. Mackay	Visiting Fellow	OD, DCE	NCI
J. R. Hartzler	Biological Lab. Worker	OD, DCE	NCI
C. C. Sinha	Biologist	OD, DCE	NCI

Objectives:

We have previously reported that the metastatic phenotype and type IV collagen degrading activity can be simultaneously turned on in NIH/3T3 cells when transfected with activated ras oncogene. Here the major objective was to examine the relationship between ras expression, collagenase activity and tumor progression in two animal models. In one model which produced experimental metastases in nude mice, the ras expression could be amplified through dexamethasone treatment in NIH/3T3 cells transfected with v-H-ras and a glucocorticoid promoter (433 cells). The second model of NMU-induced metastatic mammary carcinomas involved autochthonous and transplantable tumors possessing activated H-ras with a point mutation in the twelfth codon. This mammary carcinoma model which consists of both metastatic and nonmetastatic tumors provides the opportunity to study whether tumor progression is associated with ras amplification or increased expression. Furthermore, the effect of enhanced ras expression on metastatic capability can be evaluated in NIH/3T3 cells cotransfected with v-H-ras and a glucocorticoid promoter where up to a 20-fold increase in P21 levels can be obtained through treatment with the glucocorticoid dexamethasone.

Methods Employed:

1. The mammary tumors (usually several per animal) were induced through a single injection of NMU (30 μg/g body weight) into Sprague Dawley or Buffalo rats at 50 days. Tumors were observed in about 90% of the rats within 3-6 months. In the majority of the rats the mammary tumors did not produce metastases during the observation period of 9-12 months. However, through collaboration with Dr. Talmadge (FCRF, Frederick, MD) and Dr. Gullino (NCI) several metastatic NMU-induced tumors were obtained. Three of these tumors persistently produced the same patterns of metastases when transplanted into syngeneic rats. Comparison of individual metastases with a parent primary mammary tumor was accomplished through resection of ten separate lung metastases and a primary tumor from a rat treated with NMU 9 months earlier. The tumors were then transplanted subcutaneously into nude mice for expansion and nucleic acid isolation.

2. DNA and RNA were extracted from the NMU-induced tumors and normal rat mammary glands. For detection of H-ras-specific sequences, DNA was digested with Bam-Hl or Hind III, electrophoresed through 0.8% agarose gels and transferred to nitrocellulose filters by the Southern blotting technique. The filters were hybridized with 10^6cpm/ml of nick-translated ^{32}P-labelled c-H-ras probe or a 19-mer oligonucleotide probe possessing the NMU-induced G-A transition in the second nucleotide of codon 12. The blot hybridization mixture contained 3 x SSC, 0.05 M Tris (pH 7.5), 5 x Denhardt's solution, 1 mM EDTA and 50% formamide. The blots hybridized with the ras probe were washed three times in 2 x SSC, 0.1% SDS at room temperature and three times in 0.1 x SSC, 0.1% SDS at 60°C. The blots hybridized with the oligonculeotide probe were washed four times with 6 x SSC at 66°C for 30 min. The blots were then dried and exposed to Kodak XR-5 film at -70°C for 1-2 days. Slot blot analysis was performed on ten μg of denatured DNA or RNA from the NMU-induced tumors which were passed through nitro-cellulose filters and hybridized with a ^{32}P-labeled c-H-ras probe as described for Southern blot. Quantitation of ras-specific sequences was made with densitometric tracings and expressed as relative density units.

3. Assay for type IV collagenolytic activity in culture supernatants and tissue lysates. The 433 cells were washed three times with PBS, and incubated in serum-free medium which was collected after 48 hours and concentrated through ammonium sulfate (0-60%) precipitation, then dissolved and dialyzed against 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 5 mM $CaCl_2$ overnight at 4°C. Tissue lysates were prepared by pulverizing the frozen tissues in a mortar and homogenized by douncing in a lysate buffer composed of 0.05 M Tris-HCl (pH 7.4), 0.1 M $CaCl_2$ and 0.25% triton X-100. The homogenates were centrifuged at 10,000 rpm for 10 minutes, and the supernatants dialyzed against the same buffer (see above) as used for the culture supernatants. The tissue lysates were stored at -70°C until assayed.

   Biosynthetically [^{14}C] proline-labeled EHS type IV collagen was used as a substrate in the assay measuring collagen IV degrading activity. The samples were first incubated with trypsin (10 μg/ml) for 10 minutes at 37°C, and then soybean trypsin inhibitor (50 μg/ml), N-ethylmaleimide (3.8 mM) and aprotinin (1000 kallikrein inhibiting units/ml) were added and the mixture incubated for 16 hours at 37°C. Parallel samples were assayed in the presence of 10 mM EDTA to ensure that only the metalloproteinase activity was tested. The reaction was terminated by adding trichloroacetic acid (0.6%) - Tannic acid (0.03%) and placing the samples on ice for 30 minutes. The undigested substrate was removed by centrifugation and the radio-activity of the supernatants was measured in a β-scintillation counter. The enzyme activity was expressed either as cpm/10^7 cells or cpm/mg protein.

4. Gelatin zymograms. Metalloproteinase activity was assessed using SDS gels that contained gelatin substrate copolymerized with acrylamide. Preparation of the gel and the running buffer was the same as for regular SDS gels except for the addition of 0.1% gelatin to the 7.4% acrylamide separating gel. The electrophoresis was performed under nonreducing conditions at a constant current of 9 mA at 4°C. Samples of serum-free culture supernatants, containing equal amounts of protein (5 μg), were analyzed. Following electrophoresis the gels were washed three times in 50 mM tris-HCl (pH 7.4) containing 2% Triton X-100 for 30 min, and three times in 50 mM tris-HCl (pH 7.4) for 5 min. After rinsing the gels were incubated in a buffer containing 50 mM tris-HCl (pH 7.4), 0.2 M NaCl, 5 mM $CaCl_2$, 0.01% Triton X-100, 0.02% NaN_3 hr at 37°C for 3 H or overnight, depending on the intensity of the gelatinolytic activity. The gelatin gels were stained and fixed for 1 hr shaking at room temperature using 0.1% amido black in a mixture of acetic acid:methanol:water (1:3:6) and then destained in the same mixture without amido black.

5. Attachment assay. The effect of dexamethasone treatment on attachment of the 433 cells to different substrates was studied in vitro. Tissue culture wells were coated with type I collagen, type IV collagen, laminin or fibronectin, each substrate 10 μg/well. The 433 cells with and without six days of treatment with dexamethasone (2×10^{-6}M) were labeled with Tran ^{35}S amino acid mixture (10 μCi/ml) for 40 hr, washed and 10^6 cells added to each well. At 15 min, 30 min, 1 hr, 2 hr, 3 hr, and 6 hr the medium was removed and the wells washed gently with PBS. The attached cells were lysed with 0.2 N NaCl, 10 mM tris-HCl (pH 8.0), 0.1 M EDTA (pH 7.8) and 1% SDS and the radioactivity measured in a scintillation counter.

Major Findings:

1. Both RNA and DNA ras expression varied significantly in a group of ten individual lung metastases, derived from a single NMU-induced primary tumor. Ras expression in three of the metastases was much lower than in the parent tumor.

2. After multiple passages in syngeneic rats more uniform ras levels were observed both among the primary tumors and the metastases from different sites.

3. There was no relationship between ras-specific DNA levels and the degree of malignancy in the NMU-induced rat mammary tumors.

4. Dexamethasone treatment of 433 cells resulted in greatly increased p21 levels demonstrated by immunoperoxidase staining using sheep polyclonal antibody to p21. Nevertheless, the dexamethasone treated cells were 2.5-fold less metastatic than the untreated control. This difference could not be explained by a difference in growth rate

in vitro or in vivo. Cellular adherence to collagen I, IV and laminin was not altered by the dexamethasone treatment, but adherence to fibronectin was decreased. Furthermore, the basement membrane degrading activity of the 433 cells was decreased two-threefold by the dexamethasone treatment. This was demonstrated through a tube assay of type IV collagen degrading activity and gelatin zymograms.

5. Type IV collagenolytic activity was significantly higher in the autochthonous primary tumor and metastases than normal mammary glands.

6. There was no difference in type IV collagenolytic activity between metastatic primary tumor, nonmetastatic tumors, and metastases.

7. Gelatin zymograms of the breast tumors revealed two major metalloproteinase enzymes of 67 kDa and 92 kDa, but only the 67 kDa enzyme was visible in the normal mammary glands. Cell lines expressed similar gelatinolytic patterns as the tumors they were derived from.

Publication:

1. Thorgeirsson UP, Turpeeniemi-Hujanen T, Ballin M, Liotta LA. Methods to study ras oncogene-mediated induction of the metastatic phenotype. In: Kaiser H, ed. Progressive stages of malignant and neoplastic growth. Kluwer Academic Publishers, 1989;113-19.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05578-02 OD

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Tumor-Endothelial Cell Interaction; Basement Membrane Degradation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
Others:	M. Ballin	Visiting Fellow	OD, DCE	NCI
	J. R. Hartzler	Biological Lab. Worker	OD, DCE	NCI
	C. C. Sinha	Biologist	OD, DCE	NCI

COOPERATING UNITS (if any)

LAB/BRANCH
Division of Cancer Etiology

SECTION
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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.75	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Basement membrane (BM) dissolution is a prerequisite for tumor cell extravasation. This requires proteolytic degradation of the major structural components of BM, type IV collagen. The efficiency of this proteolytic process may depend on a multitude of cellular and tumoral factors. We examined here the possibility that interaction between tumor cells and endothelial cells may either facilitate or inhibit tumor cell extravasation through modulation of proteinase activity. Endothelial cell collagenase and tissue inhibitor of metalloproteinase (TIMP) genes were cloned from a human endothelial cell cDNA library. Both clones were found to be identical to the published sequence of the fibroblast mammalial collagenase and TIMP clones. These clones were used to study the expression of collagenase and TIMP genes in cultured endothelial and tumor cell lines under different conditions. Exposure of tumor cells to conditioned medium from endothelial cells resulted in reduction in metalloproteinase activity of the tumor cells. When microvascular endothelial cells were exposed to conditioned medium from tumor cells, the endothelial TIMP mRNA levels were increased. Medium from normal cells had no effect. This may serve as a defense mechanism for endothelial cells to prevent tumor cell degradation of BM components. Studies on the effect of transforming growth factor beta on collagenase and TIMP gene expression revealed that transforming growth factor-beta inhibited collagenase mRNA levels in normal fibroblasts, endothelial cells, and tumor cells.

To continue this study we will specifically focus on metalloproteinase and TIMP expression in tissue sections showing vascular invasion, using the in situ hybridization technique.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. P. Thorgeirsson	Expert	OD, DCE	NCI
M. Ballin	Visiting Fellow	OD, DCE	NCI
J. R. Hartzler	Biological Lab. Worker	OD, DCE	NCI
C. C. Sinha	Biologist	OD, DCE	NCI

Objectives:

Cancer cells must attach to the vascular endothelium before they can successfully exit the circulation. Since extravasation is possibly the single most important step in the metastatic process, this encounter of malignant cells with endothelial cells may be of major importance in determining the outcome of the metastatic process. This study focuses on the BM degrading proteinases and their inhibitors, released by malignant cells and endothelial cells. It has been well established that malignant cells elaborate a variety of proteinases to facilitate their penetration through connective tissue barriers. Activated endothelial cells are also capable of releasing BM degrading proteinases during angiogenesis. Although the mammalian collagenase and TIMP genes have been cloned from fibroblasts, it was not known whether endothelial cell collagenase and TIMP genes were identical to the ones isolated from fibroblasts. In addition to the cloning of these genes, the questions asked in this study were: 1) do endothelial cells block tumor cell metalloproteinases and thus prevent them from penetrating the vascular wall; 2) do endothelial cells vary in their inhibitory capacity, depending on their organ of origin and do specific tumor cells possess an escape mechanism from the inhibitory effects of the endothelial cells; 3) will tumor cell attachment to vascular endothelium lead to activation of endothelial cells which would make the endothelial cells capable of degrading their own BM, and thus assist the tumor cells in their effort to extravate; and 4) do growth factors modulate the proteolytic activity which is expressed during tumor-endothelial cell interaction.

It is important to have both an *in vivo* and *in vitro* approach to pursue this complicated cellular interaction. For the *in vitro* studies we use commercially available human microvascular and umbilical vein endothelial cells, as well as a variety of human tumor cell lines. Endothelial cells from nonhuman primates are also being harvested from fresh autopsy material. The *in vivo* studies involve histological sections from carcinogenically induced monkey tumors, both primaries and metastases. To follow the time course of vascular invasion by tumor cells in experimental metastasis assays, we will inject pigmented B16 melanoma cells into the tail vein of nude mice and study proteinase and TIMP expression in frozen sections of lungs at different time points using *in situ* hybridization. The future goal for this project is to

understand which factors are involved in determining whether or not cancer cells will attach to the endothelium, replicate in the vascular lumen, and eventually break through the vascular wall to grow as a metastatic deposit.

Methods Employed:

1. Cloning of Endothelial Cell Collagenase and TIMP Gene. The human endothelial cell lambda gt 11 cDNA library was screened with a) a human mammalian collagenase probe of 1.7 kbp or b) a 30-mer oligonucleotide probe derived from the published cDNA sequence of fibroblast TIMP gene. For the primary screening, 500,000 plaques were used and hybridized with the ^{32}P-labeled collagenase probe on the TIMP oligonucleotide probe. Plaque purified clones were isolated after three or four rounds of screening. All the clones were subcloned into pGEM 3Z for sequencing and generation of riboprobes for in situ hybridization. The Sanger technique was used for DNA sequencing.

2. Isolation of Microvascular Endothelial Cells from Omental Fat. Bacterial collagenase solution (0.1%) in Hanks balanced salt solution was mixed with minced up pieces of omentum and incubated in a shaking water bath at 37°C for 15 minutes. The mixture was filtered through a Nylon mesh with a pore size of 120 micron. The filtrate was then centrifuged at 300 g for 10 min and the supernatant filtered through a nylon mesh of 80 micron pore size. The filtrate which contains the endothelial cells was centrifuged and the pellet resuspended in the endothelial cell culture medium, consisting of M199 with 10% fetal calf serum, a penicillin-streptomycin-gentamicin mixture and 0.2% endothelial cell growth supplement.

3. Tumor-Endothelial Cell Interaction. a) In studying the effect of conditional tumor cell medium on endothelial cell expression of collagenase and TIMP, tumor or endothelial cells were exposed to conditional medium at concentrations of 0.1, 1, 5, 10, 15, 25, 50, 75 and 100%. After 24 hours exposure to the conditioned medium the cell monolayers were washed three times and replaced with serum-free medium. At 1, 3, 6, 12 and 24 hours 100 μl samples of supernatants were collected for gelatin gel electrophoresis and type IV collagenolytic activity. In parallel experiments the effect of conditioned medium on collagenase and TIMP gene expression was studied in tumor cells and endothelial cells cultured separately or together using in situ hybridization techniques.

4. Treatment with TGF-β. Subconfluent cell monolayers were grown for 24 hours in the presence or absence of 2 mM TGF-β. RNA was isolated from exposed cells and Northern blots prepared, each lane containing 1 μg of mRNA.

Major Findings:

1. Three cDNA clones of endothelial cell collagenase of 1.7, 0.9 and 0.7 kbp were isolated from a human endothelial cell library. DNA sequencing of all three clones revealed full homology with the known human fibroblast mammalian collagenase cDNA.

2. A 0.7 kbp TIMP cDNA clone was isolated from a human endothelial cell library. Similarly, the DNA sequence of TIMP was identical to the known fibroblast TIMP cDNA sequence.

3. Gelatinolytic activity of tumor cells was reduced in a dose dependent manner when treated with endothelial cell conditioned media. No effect was observed when tumor cells were incubated with normal fibroblast conditioned media.

4. After endothelial cells were exposed to conditioned medium from tumor cells (A2058) a slight increase in their gelatinolytic activity was observed.

5. In situ hybridization studies demonstrated greatly enhanced TIMP gene expression in microvascular endothelial cells after exposure to conditioned medium from tumor cells. Normal fibroblast conditioned medium had no effect on endothelial TIMP expression.

6. TIMP and mammalian collagenase mRNA levels were reduced in human microvascular endothelial cells and normal human embryonic fibroblasts following TGF-β treatment.

7. TGF-β had no effect on tumor cell (A549, HT1080) mammalian collagenase mRNA levels, but reduced the TIMP mRNA levels in both cell types.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05608-01 OD

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Purification and Further Characteristics of 92kDa Gelatinolase

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
Others:	A. R. Mackay	Visiting Fellow	OD, DCE	NCI
	M. Ballin	Visiting Fellow	OD, DCE	NCI
	R. Parker	Expert	OD, DCE	NCI
	J. R. Hartzler	Biological Lab. Worker	OD, DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A novel 92kDa type IV collagen degrading enzyme was observed to be associated with the malignant phenotype. This enzyme was semipurified by two different procedures and its activity was compared to that of 67kDa gelatinase/type IV collagenase. Both enzymes exhibited similar substrate specificities degrading human placental type IV collagen, human type III collagen, bovine type 1 collagen, and gelatin. Both enzymes exhibited a similar size reduction following incubation with the organic mercurial compound APMA, a substance reported to activate collagenases. Both enzymes were released in a latent form from cells in culture. However, true activators of these enzymes remain to be elucidated.

Purified 92kDa enzyme obtained from HL60 cell culture supernatant was found to be N terminal blocked during protein sequence analysis. Studies are underway to obtain protein sequence data from APMA generated fragments of the enzyme. Polyclonal antibodies against the 92kDa enzyme are presently being generated. Once protein sequence data and antibodies are obtained it is proposed to obtain cDNA clones encoding the 92kDa enzyme.

Other cDNA clones isolated in this group include type 1 collagenase, 67 kDa type IV collagenase and tissue inhibitor of metalloproteinases (TIMP). These clones will be used to further study the relative importance of these metalloproteinases and their inhibitor in both tumor invasion and metastasis in vivo and tumor cell interactions with endothelial cells in vitro.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. P. Thorgeirsson	Expert	OD, DCE	NCI
A. R. Mackay	Visiting Fellow	OD, DCE	NCI
M. Ballin	Visiting Fellow	OD, DCE	NCI
R. Parker	Expert	OD, DCE	NCI
J. R. Hartzler	Biological Lab. Worker	OD, DCE	NCI

Objectives:

1. To further characterize 92kDa gelatinase/type IV collagenase with respect to its substrate specificity, activation and relationship to 67kDa gelatinase/type IV collagenase.

2. To purify the enzyme.

3. To obtain protein sequence data for use in cDNA cloning.

4. To obtain polyclonal antibodies for use in cDNA cloning and immunostaining of tissue sections.

5. To obtain cDNA clones for use in transfection studies and in situ hybridization studies.

Methods Employed:

1. Cell cultures. HL60 cell line was used for the production of large quantities of culture supernatant for enzyme purification. Cells were grown in suspension in Dulbecco's modified Eagle's medium, containing 10% FCS and antibiotics. Prior to collection of supernatant cultures were incubated in serum free medium for 48 hours.

2. 92kDa enzyme was purified from ammonium sulphate precipitated culture supernatant by either a) incubation with RCA agarose 120 overnight at 4°C, followed by serial elution with increasing salt concentrations up to 1 Molar, or b) incubation with gelatin conjugated with sepharose 4B at 4°C overnight followed by elution with 5% DMSO. Enzyme activity was checked by substrate gel electrophoresis. Purity of the enzyme was checked by 2 dimensional gel electrophoresis.

3. Substrate gel electrophoresis. Preparation of the gel and running buffer was the same as for regular SDS PAGE gels except for the addition of 0.1% gelatin, type I or III collagen or 0.4% type IV collagen to the 7.5% acrylamide separating gel. Electrophoresis was carried out under nonreducing conditions. Gels were rinsed in 2% TX100, then incubated with 50 mM Tris, 0.2 M NaCl, 5 mM CaCl at pH 7.4 at 37°C. Enzyme activity was observed by negative staining following incubation with 1% amido black in acetic acid:methanol:water (1:3:6) and destaining in the same mixture without amido black.

4. Protein sequencing was carried out on purified protein transferred to immobilon filters following SDS PAGE electrophoresis on 7.5% Lemmlie gels.

5. Antibodies are being prepared in male New Zealand rabbits by subcutaneous injection initially of 1 mg of gel purified 92k enzyme. Injections were repeated at weekly intervals for the next three weeks. Antibodies will be purified from whole blood.

Major Findings:

1. 92kDa gelatinase/type IV collagenase is associated with the malignant phenotype.

2. Semipurified 92kDa enzyme has a similar substrate specificity to the 67kDa gelatinase/type IV collagenase reported by others. In substrate gels both enzymes degrade human type III and IV collagen, bovine type I collagen and gelatin to similar degrees.

3. 92kDa enzyme shows a reduction in size following incubation with the collagenase activator APMA.

4. Both 92kDa and 67kDa enzymes are released in latent form. The activators of these enzymes remain to be thoroughly elucidated.

5. RCA purification of culture supernatant separated 67 and 92kDa activity at high salt elution. Purification using gelatin sepharose resulted in a semipure preparation that when run on a 2 dimensional gel revealed no contamination at 92 kDa, but several contaminating proteins at 67kDa.

6. Protein sequencing information indicated that the 92kDa enzyme was N-terminal blocked. Studies are underway to obtain sequence from APMA treated protein fragments.

Publications:

1. Ballin M, Gomez DE, Sinha CC, Thorgeirsson UP. Ras oncogene mediated induction of a 92 kDa metalloproteinase strong correlation with the malignant phenotype. Biochem Biophys Res Commun 1988;154:832-8.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05609-01 OD

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gelatinase / Type IV Collagenase Response in Normal and Neoplastic Cells to TPA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	U. P. Thorgeirsson	Expert	OD, DCE	NCI
Others:	A. R. Mackay	Visiting Fellow	OD, DCE	NCI
	M. Ballin	Visiting Fellow	OD, DCE	NCI
	J. R. Hartzler	Biological Lab. Worker	OD, DCE	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.75	0.25

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided)*

Two metalloproteinases of 67kDa and 92kDa capable of degrading a variety of collagenous substrates, including type IV collagen, are under investigation. Both have been related to the malignant phenotype and may play important roles in tumor cell invasion and metastasis.

The tumor promoting phorbol ester TPA was observed to have divergent effects on the expression of these enzymes when normal cell lines were compared to neoplastic cell lines. Normal cells expressing 67kDa enzyme activity showed little TPA responsiveness. Tumor cell lines expressing 67kDa activity but low 92kDa activity showed a marked selective increase in 92kDa activity following TPA treatment. Other tumor cell lines already expressing high levels of both 67 and 92kDa activity showed little TPA responsiveness. This would not only suggest a difference in the regulation of the two enzymes, but would also suggest that some cells are predisposed to produce the 92kDa enzyme upon receiving the right stimulus. TPA activates protein kinase C (PKC) and has been reported to induce activation of proto-oncogenes jun and fos in certain cell lines, which in turn form a complex capable of activating AP1 responsive genes. Analysis of nuclear protein jun/fos complexes and activated PKC is under investigation in these cell lines. Members of the metalloproteinase family of enzymes, namely type 1 collagenase and stromelysin have already been shown to have TPA responsive regulatory elements with AP1 binding DNA sequence. Although regulatory DNA sequence data is not available for either 67kDa or 92kDa enzymes, cDNA and amino acid sequence data for the 67kDa enzyme show close homology to both type 1 collagenase and stromelysin. These experiments may provide some insight into the regulation of these important enzymes involved in tumor cell invasion and metastasis.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

U. P. Thorgeirsson	Expert	OD, DCE	NCI
A. R. Mackay	Visiting Fellow	OD, DCE	NCI
M. Ballin	Visiting Fellow	OD, DCE	NCI
J. R. Hartzler	Biological Lab. Worker	OD, DCE	NCI

Objectives:

1. To investigate whether enzymes capable of degrading type IV collagen, possibly linked to the malignant phenotype, can be induced in "normal" cells by the tumor promotor TPA.

2. To investigate the effect of TPA on neoplastic cells with respect to two enzymes with type IV collagenolytic activity.

3. To assess the levels of AP1 binding jun/fos complexes in normal and tumor cells following TPA treatment and to relate this to the expression of enzymes capable of degrading type IV collagen.

4. To assess the levels of activated PKC in control and TPA treated cells.

Methods Employed:

1. Cell cultures. Cell lines stored in liquid nitrogen were grown in their respective recommended media containing antibiotics. Routinely, cells were grown on 150 mm petri dishes. Upon reaching subconfluency cells were washed three times in PBS then incubated in the absence of serum with 1 x 10^{-8} M TPA (16 plates) or with medium alone (16 plates). Culture supernatants and cells were harvested separately at 6, 12, 24 and 48 hours following TPA addition. Lysates were prepared from the cell pellets for a) enzyme activity, b) jun/fos complex assay, and c) PKC activity. Cell supernatants were used for enzyme activity.

2. Cell lysates. Cell lysates were prepared in three ways: a) for gelatinase activity cell pellets were rinsed in PBS three times. Pellets were frozen and thawed three times at -70°C. Cells were then resuspended in an equal volume of 50 mM Tris HCl 1% TX100 (pH 7.5). Cells were sonicated for 5 seconds at level 4. Cell debris was removed and the lysates stored at -80°C; b) for PKC activity, cell pellets were washed in PBS 10 mM EDTA three times, resuspended in an equal volume of 50 mm Tris 1% TX100 containing protease inhibitors leupeptin, PMSF, aprotinin, pepstatin, DTT and soybean trypsin inhibitor. Cells were sonicated for 5 seconds at level 4. Cell debris was removed and lysates stored in aliquots at -80°C; and c) for jun/fos, cell pellets were washed three times in PBS. Pellets were frozen and thawed at -70°C. Pellets were resuspended in an equal volume of buffer C containing PMSF and DTT. Cell debris was discarded and the lysates stored at -80°C.

3. Substrate gel electrophoresis. Preparation of gel and running buffer was the same as for regular SDS PAGE gels except for the addition of 0.1% gelatin or 0.4% type IV collagen to 7.5% acrylamide separating gels. Electrophoresis was carried out under non-reducing conditions. Gels were washed in 2% Triton X-100 to displace SDS, rinsed with 50 mM Tris (pH 7.5) and incubated at 37°C for 16 hours in 0.05 M Tris, 0.2 M NaCl, 5 mM CaCl, 1% TX100 (pH 7.4). Enzyme activity was visualized by negative staining after incubation of the gels with 1% amido black in acetic acid:methanol: water (1:3:6) and destained in the same mixture without amido black.

4. Western blot. To determine PKC content and the amount of fos and jun in cell lysates, protein estimates of cell lysates were performed and equal amounts of protein loaded onto SDS PAGE gels. Proteins were transferred onto nitrocellulose and western blots were performed using antibodies against PKC, jun and c-fos.

5. Gel retardation assays were performed using type 1 collagenase and stromelysin AP1 binding site oligonucleotides for the determination of active jun/fos complexes. 4 μg cell lysates were mixed with 0.4 ng of P32 end-labelled oligonucleotide plus 3 μg of poly DIC to remove nonspecific protein binding in buffer D plus 50 mM NaCl. Samples were incubated for 20 minutes and run on 4% DNA acrylamide gels. DNA shifts were assessed following autoradiography.

Major Findings:

1. Normal human diploid fibroblasts already expressed 67 kDa type IV collagenolytic activity and were unresponsive to TPA stimulation.

2. TPA markedly enhanced 92 kDa gelatin/type IV collagenolytic activity with little effect on 67 kDa activity in cell lines initially expressing low levels of 92 kDa activity.

3. TPA had little effect on cell lines already expressing high levels of 92 kDa activity.

4. Initial observations may suggest correlation of 92 kDa expression with the presence of active jun/fos complexes, but this is to be confirmed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05621-01 OD

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The Involvement of ras Oncogenes in Chemical Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. J. Parker	Expert	OD, DCE NCI
Others:	S. M. Sieber	Deputy Director	OD, DCE NCI
	R. H. Adamson	Director	OD, DCE NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Division of Cancer Etiology

SECTION
Office of the Director

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.25	0.25	0.00

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Activated oncogenes have been identified in a wide range of human neoplasms. In rodents, the ras family of oncogenes are activated by single point mutations which arise following the interaction of DNA with certain chemical carcinogens. DNA from tumor tissues collected over a period of 25 years from nonhuman primates on long-term treatment with a variety of chemical carcinogens will be examined for the presence of activating point mutations in the N-, K- and H-ras loci. Initial studies indicate a very high degree of homology (100%) between man and monkey with respect to nucleotide sequences in normal DNA at the N- and K-ras loci and greater than 95% homology at the H-ras locus. Using the polymerase chain reaction (PCR), as little as 0.1 ng of DNA isolated from 3-micron sections of paraffin embedded tissue could be amplified at any of the N-, K- or H-ras loci and their nucleotide sequence obtained following subcloning. These techniques will allow rapid screening of tumor DNA for activating ras mutations in formalin fixed tissue samples.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. J. Parker	Expert	OD, DCE	NCI
S. M. Sieber	Deputy Director	OD, DCE	NCI
R. H. Adamson	Director	OD, DCE	NCI

Objectives:

The major goal of this project is to study the role of oncogene activation in the development of chemically induced tumors in nonhuman primates. The frequency of activating point mutations in the ras locus is being measured in DNA isolated from tumors induced by several different chemical carcinogens including 2-amino-3-methylimidazo-[4,5-f]quinoline (IQ) and N-nitroso-N-methylurea (NMU).

Methods Employed:

1. Formalin-fixed autopsy samples of normal and tumor tissues previously taken from monkeys on long-term treatment with a vartiety of chemical carcinogens were paraffin-embedded and serial sectioned. Sections were either stained for histopathological evaluation or used for DNA extraction.

2. The genomic DNA extracted from each tissue section was used as a template for PCR amplification of specific DNA fragments corresponding to codons 12 or 61 of the N-, H- and K-ras oncogenes.

3. The PCR amplified fragments were purified on agarose gels and subcloned in E. coli using Bluescript phagemid as a vector.

4. For each PCR fragment, 12 insert-containing clones were cultured and each sequenced by standard dideoxy sequencing procedures.

Major Findings:

Preliminary findings indicate an absence of activating point mutations in either the H-, K- or N-ras loci of DNA isolated from a single cynomolgus monkey that had been on long-term treatment with IQ. The nucleotide sequences in the region of the K-, and N-ras codons 12 and 61 in DNA obtained from both liver tumor and normal monkey liver were found to be identical to the published human sequences. H-ras nucleotide sequences in DNA from both liver tumor and normal monkey liver showed only minor differences to the corresponding human sequences. These differences were found in codons 6, 27, 28, 57, 58 and 61 of H-ras and were, in all cases, third base substitutions that would not affect amino acid sequence and are thus insignificant in terms of oncogene activation.

ANNUAL REPORT OF

THE BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 through September 30, 1989

The Biological Carcinogenesis Program (BCP) (1) plans, develops, directs and evaluates a national extramural and intramural research program in which the role of biological agents, genetic sequences, viral genes, and combinations of viral and cellular genes in carcinogenesis is investigated; supports efforts to isolate and analyze proteins responsible for transformation, to identify genetic sequences coding for these proteins, to investigate transforming growth factors and mechanisms of transformation, and to study the role of RNA- and DNA-containing viruses in certain forms of cancer; (2) develops improved management methods and practices, and maintains liaison with intramural and extramural scientists, as well as various extramural organizations and scientists; (3) assists in fostering new research and resource programs based on intramural and extramural research requirements and new research leads; and (4) assists in the allocation of resources and establishes program priorities for these activities.

The research and other activities of each Laboratory and Branch are described in the sections following this report, which focuses on the highlights of the overall Program.

Intramural

Virus Studies: HIV

Spindle-like cells were successfully cultured for long periods of time from tissue specimens taken from the lung, mouth, skin, orbit, tonsil, and pleural effusion obtained from acquired immunodeficiency syndrome (AIDS) patients with disseminated Kaposi's sarcoma. Functional studies of these cells with respect to their role in matrix formation or destruction with the help of collagenases, chemotactic and chemoinvasion capabilities have just been completed. The apparent conclusion of this study seems to be that these cultured cells are abnormal smooth muscle cells of vascular origin. Monocyte/macrophages (M/M) have been shown to be highly susceptible and permissive host cells for human immunodeficiency virus type 1 (HIV-1). This cell system can be successfully used for virus isolation in situations when the T-cell system fails. HIV-1 has been isolated from M/M from sero-negative contacts of HIV-1 infected individuals (e.g., spouses) but not from T-cells. Nucleic acid analysis of these new isolates has shown them to have unique banding patterns when hybridized to molecular probes of HIV-1. Studies are continuing to define the level of HIV-1 expression in M/M infected with these "low" replicative isolates as compared to "high" replicative isolates.

Several drugs have been examined either alone or in combination for inhibition of HIV-1 replication in cell cultures. Drugs that inhibit virus replication by mechanisms other than reverse transcriptase inhibition and DNA chain termination may be useful in the treatment of patients with AIDS and AIDS-related complex (ARC). The following drugs have been studied: D-penicillamine, amphotericin, and Avarol with AZT.

Several approaches to the development of an AIDS vaccine are being pursued. The potential usefulness of synthetic peptides as candidate AIDS vaccines has been explored, especially a peptide generated from the p17 sequence on the surface of HIV-1. Because of the genetic variability in the gp120 region of different HIV-1 isolates, which show as much as 20% divergence in the amino acid sequence of gp120, it is important to look for approaches other than the conventional aim of a gp120 vaccine in the development of an AIDS vaccine.

A 30-amino acid peptide analog of HIV-1 p17 (termed HGP30) and other analogs spanning the HIV-1 p17 amino acid sequence have been synthesized that cross-react in the HIV-1 p17 radioimmunoassays (RIA) and can be identified by HIV-1 p17 monoclonal antibodies by enzyme linked immunosorbent assays (ELISA) and Western blotting. Antibodies prepared against HGP30 inhibit HIV-1 replication in cell culture. These studies indicate that HIV-1 p17 may be important in immunity to HIV-1 and that the antibodies may be protective against HIV-1 infection. Sera of 9% of AIDS patients (7/76) and 18% of HIV-1 seropositive healthy homosexuals (40/223) were positive for HGP30 antibodies. Decline in HIV-1 p17 antibodies has been shown to be related to disease progression in both children and adults. A limited clinical trial with HGP30 is currently in progress in England.

Results of a six-year prospective study of a cohort of HIV-1-seropositive homosexual men have shown that neutralizing antibodies are correlated with long periods of disease-free status. This observation confirmed and extended earlier findings of an association of neutralizing antibodies with a better clinical outcome in both adult and pediatric patients. Nevertheless, long-term prospective studies of seroconverters will be necessary to establish whether neutralizing antibodies are truly protective, with high titer antibodies correlated with longer time to AIDS onset and prolonged survival. On-going studies of the humoral immune responses in HIV-seropositive mothers and their children will help establish which responses are protective and associated with healthy, HIV-negative offspring.

The neutralizing antibody profile showed a plateau level of moderate neutralizing antibody titer which continued for several years. Then, following a marked increase in titer observed over a two-year period, neutralizing activity declined prior to AIDS diagnosis. To better understand the factors contributing to this profile, a molecular epidemiologic study has been initiated. Following extraction of RNA from stored lymphocytes of the same individuals followed for six years, reverse transcription and subsequent polymerase chain reaction will allow analysis of the levels of HIV gene expression during the course of disease progression. In particular, the envelope and regulatory genes are being studied. Results will be analyzed with regard to the immune responses already known for the study subjects, and their overall clinical and immunologic status.

Using a monoclonal antibody an HIV neutralizing epitope has been mapped to a 24-amino acid region within the viral envelope. Others, using alternate techniques, have mapped the same site, now recognized as a major type-specific immunodominant epitope. This site is being exploited extensively, with the hope that it will provide at least one component of a subunit vaccine. As this region is very heterogeneous, it is important to know the degree of variability among a number of viral isolates and the range of cross neutralization elicited by specific sequences.

Studies have been continued on the structure-function relationship of HIV-1 trans-regulatory genes with an emphasis on virus life cycle. In an early study based on site-directed mutagenesis, it was shown that the cysteine residues in the tat trans-activator protein are essential for virus expression. This region of tat is proposed to have a possible role in metal-nucleic acid binding or dimerization. Recent studies have shown that conserved missense mutations with predicted cysteine to histidine changes within the proposed tetrahedral structure of the nucleic acid binding motif eliminate tat activity and virus expression. In contrast, one cysteine-to-histidine mutation outside the putative tetrahedron had little effect on virus expression. Although all but one cysteine residue in this domain are important for tat function, the region is unlikely to assume a prototype zinc-finger structure.

The rev trans-regulator protein, though essential for virus replication, appears to play a pivotal role in the balance of HIV-1 gene over- or underexpression. Last year we reported that a rev-defective mutant HIV-1 expresses significantly higher levels of nascent viral RNA than wild type. The rev, like nef, may possibly play a negative role in virus transcription. Extension of these studies clearly show that rev exerts both a positive and a negative effect on virus replication, depending on the relative amount of rev supplied in trans.

In a prospective study, HIV-1-infected individuals with the HLA-DR1 phenotype were found to progress to clinical disease more rapidly than did individuals with other major histocompatibility complex class II antigens.

Virus Studies: Human Tumor Lympotropic Virus Type I (HTLV-I)

HTLV-I has been studied as the etiologic agent of adult T-cell leukemia. Studies have shown that trans-activation of HTLV-I LTR by the virus-coded trans-activating protein Tax1 is correlated with the presence of a cAMP-responsive octonucleotide. It has also been established that two signal transduction agents, cAMP and TPA1, are both potent activators of the HTLV-I LTR. The cAMP-responsive and the TPA-responsive sequences have been shown to be the HTLV-I LTR.

It has been shown that the Tax1 protein binds specifically to a zinc affinity column. The putative metal binding domain of Tax1 may play an important role in trans-activation. Since Tax1 appears not to bind DNA directly, its interaction with cellular transcription factors may be facilitated by coordinating a common zinc ion.

Three lines of transgenic mice expressing the HTLV-I tax1 gene were previously developed here; these mice develop neurofibromas that recruit granulocytes as a result of tumor secretion of significant quantities of granulocyte-macrophage colony stimulating factor (GM-CSF). The tumors also secrete significant quantities of nerve growth factor (NGF). This observation suggests that tax1 may stimulate NGF production and may provide important new insights into the mechanism of HTLV-I induced neurologic disease (tropical spastic paraparesis; also a controversial suggestion of multiple sclerosis).

The HTLV-I strain from CSF of a patient with tropical spastic paraparesis (TSP) shows differences from the prototype HTLV-I isolate from adult T-cell leukemia (ATL) patients by restriction enzyme mapping, suggesting that the TSP HTLV-I strain is similar, but not identical, to the prototype HTLV-I isolate. A genomic library of this HTLV-I strain has been obtained and is being subcloned for

further characterization. Several cell lines developed from TSP patients have been characterized phenotypically by using a panel of monoclonal antibodies to lymphoid cell surface markers, such as CD3, CD4, CD25, CD8, and HLA-DR.

HTLV-I seroprevalence prior to the AIDS epidemic was determined in the U.S. drug abuser populations. Samples from 1976-1980 (9,499 samples) were screened by ELISA using HIV-1-H9 or HTLV-I-HUT102. One HIV-1-positive serum from an individual with HIV risk factors was identified. Twenty individuals were seropositive for HTLV-I. None appeared to be HTLV-II specific. HTLV-I seropositivity was statistically higher in blacks (0.71%) and other minorities (1.03%) than in whites (0.12%). HTLV-I seropositivity was statistically higher for people above the age of 45 years. The data indicate that HTLV-I has existed in the U.S. longer than HIV-1. These studies indicate that drug abusers (DAs) should be investigated for increased incidence of leukemia and lymphoma related to HTLV-I.

Virus Studies: Cytomegalovirus (CMV)

Cytomegalovirus (CMV) has been epidemiologically associated with several human tumors, not definitively linked to any, but is capable of transforming human cell lines. The transforming role of three different CMV strains was studied to determine the gene sequences essential for transformation. The morphological transforming region II (mtr II) of CMV Towne, a strain of CMV, has been localized to a 980-base-pair fragment containing three putative open reading frames (ORFs) of 79, 83, and 34 amino acids. The 2.2 kb colinear region in strain AD169 was also transforming, but the colinear mtrII region in the Tanaka strain had significantly less transforming potential. Analysis revealed the presence of the 79-amino acid ORF in strains Towne and AD169 but not in Tanaka. These comparative data indicate an important role for the 79-amino acid ORF in transformation by CMV.

Virus Studies: JC Virus

The JC virus (JCV) has been postulated to be involved in the formation of glial tumors in patients with progressive multifocal leukoencephalopathy. In transgenic mice incorporating the JC virus, the animals develop adrenal neuroblastomas. A cell line derived from neural crest cells has been found that is permissive for JCV. An interesting aspect of these studies has been the finding that the addition of retinoic acid, which stimulates neuronal cell differentiation, elevates the levels of JCV DNA replication and transcription.

Virus Studies: Papillomaviruses

Genetic analyses have been carried out to define the open reading frames that encode each of the three E2 proteins of bovine papillomavirus (BPV). These studies have shown that the amino terminal 200 amino acids (which are highly conserved among the E2 proteins) are necessary for the transactivator function. The transcriptional transactivator functions through direct binding to DNA sequences, and this DNA binding activity is found in the carboxy terminal 100 amino acids. A model has been created to study the factors with which E2 may interact to mediate the transcriptional activation, in which E2 proteins were introduced into Saccharomyces cerevisiae.

Continuing studies on transgenic mice harboring the complete BPV-1 genome have revealed differences in the genetic pattern in the tumors they develop. The mice are normally diploid. The benign fibroblastic proliferation (fibromatosis) that the mice develop are often aneuploid but without specific abnormalities. The fibrosarcomas, which some of the mice develop at 8-9 months of age, have consistent abnormalities in chromosomes 8 and/or 14 (not directly related to the integration of the virus which is on chromosome 15). This may be a useful model for studying the genetics of papillomavirus-induced tumors.

A quantitative in vitro keratinocyte assay for the biological activity of the papillomaviruses has been developed. Only the human papillomavirus (HPV) types associated with a high risk for malignancy are capable of altering the keratinocyte response to serum- or calcium-induced terminal differentiation; these cells extend their life span and may be immortalized. This assay has permitted identification of the two genes of HPV-16 and HPV-18 (the two HPV types associated with cervical cancer) which are both necessary and together sufficient for keratinocyte transformation. These are the E6 and E7 genes. Furthermore, the E7 protein of HPV-16 and HPV-18 have been shown to be capable of complexing with the retinoblastoma tumor suppressor gene product (p105-RB).

Virus Studies: Simian Virus 40 (SV40)

The transforming region of SV40 contains coding sequences for three proteins, large T-antigen, small t antigen, and simian virus 40 early leader protein (SELP). Analysis of SV40 T- and t-antigens suggested both proteins have domains that are similar to regions of the adenovirus E1A protein that plays a key role in regulation of transcription. It was demonstrated that t-antigen acts as a *trans*-activating protein, capable of inducing transcription from promoters that are responsive to E1A.

Virus Studies: Human Herpesvirus-6 (HHV-6)

HHV-6 may be synergistic with HIV-1. A number of cell lines consisting of T- and B-lymphocytes as well as cells of other origins, e.g., megakaryocytes, can be infected by HHV-6. Many of these same cell lines are also susceptible to infection by HIV-1. The dual infection of a susceptible cell line by HIV-1 and HHV-6 results in an accelerated loss of viable cells. While these observations are consistent with possible synergistic activity, the actual mechanisms involved are unknown. HHV-6 could contribute directly by stimulating HIV-1 expression or by interacting with HIV-1 long terminal repeat segments affecting virus expression.

The following significant developments have taken place: 1) examination of the possible role of HHV-6 in lymphoproliferative diseases and chronic fatigue syndrome; 2) study of the interaction of HHV-6, HIV-1 and HIV-2 in lymphoid cells to understand the role of this virus in AIDS; and 3) a collaborative study to develop a series of monoclonal antibodies to HHV-6 proteins.

Dual infection of CD4+ cells (fresh cells or cell lines) with HHV-6 and HIV-1 or HIV-2 showed enhanced killing, a significant increase in HIV-1 RT activity, and transactivation of HIV LTRs suggesting that HHV-6 may contribute directly or indirectly to the depletion of CD4+ T-cells in AIDS. An antigen capture assay has recently been developed as a consequence of the purification of several viral proteins and monoclonal antibodies.

HHV-6 can coinfect with HIV-1 human CD4+ T-cells leading to accelerated cell death, and factors in HHV-6 infected cells stimulate HIV-1 LTR-directed gene expression. The cis-acting sequences of HIV-1 LTR responsive to trans-activation have been localized. In addition, HHV-6 trans-activates HIV-2 and simian immunodeficiency virus (SIV) LTR, but not HTLV-I LTR. Increased HIV-1 LTR trans-activation is obtained in HHV-6-infected cells coinfected with HIV-1 or cotransfected with the HIV-1 tat gene.

Oncogenes: Platelet-derived Growth Factor, alpha and beta (PDGF-α and -ß)

A retrovirus containing the entire human PDGF-ß gene was constructed in order to investigate the in vivo biological activity of its encoded growth factor. When this virus was introduced into newborn mice, it reproducibly generated fibrosarcomas at the site of inoculation. Evidence that the normal growth factor-coding sequence was unaltered derived from RNAase protection studies and immunoprecipitation analysis. Tumors were polyclonal but demonstrated clonal subpopulations. Moreover, tumor-derived cell lines rapidly became monoclonal upon growth in culture. These findings argue that PDGF-ß overexpression can provide the initiating event for development of a clonal malignancy. Proviruses in each fibrosarcoma analyzed contained a deletion of 149 nucleotides downstream of the PDGF-ß coding region. This deletion originated from an alternative or aberrant splice event that occurred within exon 7 of the PDGF-ß gene and mimicked the v-sis oncogene. Thus, deletion of this region must confer a growth advantage to cells transformed by these retroviruses or be necessary for efficient retrovirus replication.

The transforming activities of PDGF-α and PDGF-ß genes were compared. The PDGF-α chain gene was markedly less efficient in inducing transformation than the PDGF-ß gene under the influence of the same promoter. There are significant differences in the secretory and growth stimulating properties of the two chains. These properties appear to account for the much more potent transforming ability of the PDGF-ß gene. These findings provide insights into biologic properties of a growth factor responsible for potent autocrine stimulation of abnormal cell proliferation.

A genomic sequence and cloned complementary DNA has been identified for a novel receptor-like gene of the PDGF receptor/colony stimulating factor type 1 receptor subfamily. The gene recognized a 6.4-kilobase transcript that was coexpressed in normal human tissues with the 5.3-kilobase PDGF receptor mRNA. Introduction of complementary DNA of the novel gene into COS-1 cells led to expression of proteins that were specifically detected with antiserum directed against a predicted peptide. When the new gene was transfected into COS-1 cells, a characteristic pattern of binding of the PDGF isoforms was observed which was different from the pattern observed with the known PDGF receptor. Tyrosine phosphorylation of the receptor in response to the PDGF isoforms was also different from the known receptor. The new PDGF receptor gene was localized to chromosome 4q11-4q12. The existence of genes encoding two PDGF receptors that interact in a distinct manner with three different PDGF isoforms likely confers considerable regulatory flexibility in the functional responses to PDGF.

Oncogenes: Keratinocyte Growth Factor (KGF)

A novel growth factor specific for epithelial cells was identified in conditioned medium of a human embryonic lung fibroblast cell line. The factor, provisionally

termed keratinocyte growth factor (KGF) because of its predominant activity on this cell type, was purified to homogeneity. KGF was both acid and heat labile and consisted of a single polypeptide chain of approximately 28 kilodaltons. Purified KGF was a potent mitogen for epithelial cells, capable of stimulating DNA synthesis in quiescent BALB/MK epidermal keratinocytes. Lack of mitogenic activity on either fibroblasts or endothelial cells indicated that KGF possessed a target cell specificity distinct from any previously characterized growth factor. Microsequencing revealed an amino-terminal sequence containing no significant homology to any known protein.

Oncogenes: erbB-2

Membrane protein levels of erbB-2 and EGF receptor as well as gene aberrations affecting these proto-oncogenes in human mammary cancer were determined in primary and metastitic lesions. Among 57 patients, erbB-2 gene amplification was detected in 11 tumors (19%). In 10 of these patients in whom expression levels could be assayed gene amplification was associated with a high level of erbB-2 protein. In contrast, EGF receptor gene amplification with overexpression of the protein product was observed in two tumors (4%). In addition, 14 out of 53 (26%) primary tumors exhibited moderately increased erbB-2 protein levels in the absence of gene amplification. Similar aberrations resulting in overexpression of EGF receptor protein without detectable gene amplification were associated with two tumors (4%) among 47 patients analyzed. In seven patients, expression level and gene copy numbers of erbB-2 or EGF receptor were similarly altered in the primary tumor and metastatic lesions derived from the same patient. Concordance of increased receptor gene expression in primary and metastatic lesions, combined with the observation that such alterations are detectable as early as stage I and II mammary tumors, suggests that overexpression of erbB proto-oncogenes can develop early in breast cancer and be maintained during tumor progression. Comparison of erbB-2 overexpression with clinical disease parameters revealed a correlation of this alteration with inflammatory mammary carcinoma, implying an association of elevated erbB-2 protein levels with enhanced malignancy of the tumor cell in vivo.

Oncogenes: Epidermal Growth Factor (EGF)

Alterations affecting the EGF transforming growth factor α (TGF-α)-responsive mitogenic pathway are frequently detected in malignancies. In particular, the EGF receptor (EGFR) molecule has been found overexpressed in a number of human tumors, and TGF-α is produced by a large array of tumor cells. Gene transfer experiments have previously demonstrated that expression of either TGF-α or EGFR alone is not sufficient to induce the transformed phenotype in NIH 3T3 cells. The gene for TGF-α was shown to act as a potent oncogene in NIH 3T3 cells overexpressing EGFR. TGF-α directly stimulates proliferation of the cell in which it is produced and the extracellular compartment of the transformed cell is the major site of interaction between TFG-α and EGFR. Analysis of human tumor cell lines revealed a strong correlation between expression of TGF-α and overexpression of EGFR. Moreover, high levels of EGF-independent tyrosine phosphorylation of the EGFR were detected both in NIH 3T3 cells expressing TGF-α and in high EGFR and TGF-α coexpressing human tumor cell lines. Thus, the two events instituting the EGFR/TGF-α autocrine loop responsible for transformation in vitro may play a role in the development of some human malignancies.

Oncogenes: raf

The structure-function analysis of c-raf-1 was completed, leading to the following findings: (a) c-raf-1 protein kinase is a substrate for several transmembrane and intracellular tyrosine kinases as well as for protein kinase C (PKC); (b) tyrosine phosphorylation in a specific position activates the enzyme and PKC achieves a lesser degree of activation; and (c) in the case of the PDGF receptor, tyrosine phosphorylation occurs after direct binding of c-raf-1 protein to the PDGF-activated PDGF receptor kinase domain. This is the first example of a protein kinase cascade involved in mitogen signal transduction. Activated raf, after initial translocation to the plasma membrane, moves to the perinuclear area, if not to the nucleus. Thus, raf protein kinase acts like a shuttle enzyme that connects mitogenesis-related events at the plasma membrane to events in the nucleus.

Oncogenes: myb and ets

To study the mechanism and cooperative role of the myb and ets oncogenes of the avian erythroleukemia virus, E26, in oncogenesis and to determine the biological function of v-ets and its cellular homologs in altering cell growth and hematopoietic development, novel retroviral vectors were developed containing these oncogenes that would infect murine systems to provide new biological assays that would characterize these functions. A replication-defective murine retrovirus, ME26, was constructed by insertion of the avian gag-myb-ets oncogene sequences, derived from the avian E26 leukemia virus, into an Abelson murine leukemia virus-derived retroviral vector. This novel virus induces myeloid and erythroid leukemias in newborn mice. Histologic analysis of diseased organs indicated that the majority of these leukemias (54%) were erythroid and myeloid neoplasms, while the remainder were lymphoblastic leukemias similar to that observed with helper virus alone. Leukemic cells from early cases of hematopoietic disease contain multiple copies of integrated ME26 provirus and there was evidence that at least some of the tumors were clonal in origin.

The members of the human ets gene family now account for five independent chromosomal loci: ets-1 ets-2, erg, elk-1, elk-2, and have been localized at chromosome regions, some of which are of genetic interest, both for constitutional and acquired (neoplasia) genetic disease. The ets-1 gene has been found: (1) transposed, but not structurally altered in several translocations associated with acute non-lymphocytic leukemia, and transcribed at very low, but normal-sized levels in acute non-lymphocytic leukemia (ANLL), regardless of whether they are positive for translocations; (2) neither transposed nor structurally involved in Ewing's sarcoma, neuroepithelioma, Askin's tumor with (11;22) translocation and in the constitutional (11;22) chromosomopathy. The hypothesis of a relationship between ets-2 and Down's syndrome has been tested on two grounds. While ets-2 tested negative as a putative cis-acting genetic element, not interfering with correct chromosome 21 segregation at meiosis, it was shown to be a component of the minimal genetic region responsible for Down's syndrome (DS). Increased ets-2 gene dosage, certainly extraneous to Alzheimer's disease (AD), is probably not the cause of AD developed by DS individuals. That higher ets-2 gene dosage might be responsible for the post-natal predisposition to leukemia in DS remains to be proven. To study the role of ets proto-oncogenes in the normal developmental processes and tumorigenesis, transgenic mice were generated that contain ets-2 genes.

Oncogenes: Human Gene Map

The human gene map was extended using physical mapping methods to study genes which influence human tumorigenesis. The combination of two gene mapping technologies, somatic cell hybrid analysis and *in situ* hybridization, has been used to map 35 distinct human structural genes. The rodent x human hybrid panels were developed using isozyme markers and high resolution G-trypsin banding from a group of over 1,000 hybrid clones. Southern analysis of hybrid DNA and *in situ* hybridization of tritiated probes to metaphase chromosomes have resulted in chromosomal localization of proto-oncogenes, and genes for growth factors, growth factor and retrovirus receptors, endogenous retroviral segments, retroviral integration sites, and members of the immunoglobulin gene super family.

Extramural

Oncogenes: Anti-oncogenes

A class of recessive oncogenes called anti-oncogenes or suppressor oncogenes has been discovered in which the absence of the oncogene product, rather than its presence, is responsible for transformation. One of the best understood examples of suppressor genes is the Rb gene of retinoblastoma, which appears to play an important role in the induction of retinoblastoma, as well as other commonly occurring cancers, such as cancers of the lung and colon. Loss or inactivation of both copies of the Rb gene in humans may predispose an individual to cancer. It was recently found that loss of both copies of the Rb gene in retinoblastoma tumors results in loss of cellular response to the growth inhibitory substance, transforming growth factor beta (TGF-beta). Subsequently, it was demonstrated that the mechanism for this loss of response by the tumor cells appears to result from loss of the cellular receptors for TGF-beta. These observations may explain the transformed phenotype of retinoblastoma cells.

A second observation suggests that inactivation of the Rb protein may be a mechanism of oncogenesis used by as many as three groups of DNA viruses. Adenoviruses, papillomaviruses, and polyomaviruses all encode specific viral proteins responsible for the malignant transformation of infected cells. Some of these same viral transforming proteins have been demonstrated to bind tightly to the retinoblastoma protein. Since cancer is promoted by the loss or inactivation of the Rb gene, and consequently of the Rb protein, the simplest hypothesis for oncogenesis by these viruses is the inactivation of the Rb protein as a result of complex formation with viral transforming proteins.

A presumptive second anti-oncogene has been identified in the small DNA tumor virus SV40. The cellular protein product of this anti-oncogene, designated p53, was initially recognized by its ability to bind the transforming protein of the SV40 large T-antigen. p53 was at first thought to be an oncogene, since it appeared to cooperate with the *ras* oncogene to transform primary cells in culture. Recent studies have demonstrated that the p53 DNA used in those studies represented mutant p53 which had lost its ability to act as an anti-oncogene. Experiments with unmutated normal p53 demonstrated its ability to inhibit transformation by two genes that would, in combination, normally transform cells (the adenovirus E1A gene and *ras* oncogene). One way in which SV40 T-antigen may transform cells is by binding to and inactivating the p53 protein. The study of the cellular proteins to which viral transforming genes bind may provide a useful approach to identifying additional cellular anti-oncogenes.

Virus Studies: HIV

The CD4 molecule on T-lymphocytes is the receptor for the HIV-1 and SIV viruses. Recent in vitro studies have demonstrated that the recombinant soluble form of CD4 (rsCD4) is a potent inhibitor of replication of both viruses. SIV-infected rhesus monkeys, which have a disease similar to human AIDS, received daily intramuscular injections of rsCD4 to assess the therapeutic efficacy of rsCD4 in preventing SIV infection or its associated immunodeficiency disease. Isolation of SIV from peripheral blood lymphocytes became increasingly difficult as therapy continued. However, between 60-90 days after the termination of therapy, virus could again be isolated from the animals. The rsCD4 may act by several mechanisms: by absorbing soluble SIV envelope glycoprotein and thus diverting cytolytic T-cells from killing infected lymphocytes; by inhibiting the fusion of virus infected cells with uninfected cells thus inhibiting the spread of virus; or by directly blocking the interaction of virus with CD4 molecules on target T-cells. Thus, this material could have potential value in the treatment of AIDS patients.

Virus Studies: HTLV

Studies were undertaken to determine whether HTLV augments HIV production. After mitogenic stimulation by noninfectious HTLV-I virions, peripheral blood leukocytes infected in vitro with HIV-1 produced large quantities of HIV-1. The HTLV-I virions exerted this effect prior to, immediately following, or well after the cells were infected with HIV-1. These results provide further impetus for studies of individuals infected with both viruses to determine whether HTLV-I may act as a cofactor in clinical AIDS.

ANNUAL REPORT OF

THE LABORATORY OF CELLULAR AND MOLECULAR BIOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 to September 30, 1989

The major goals of the Laboratory of Cellular and Molecular Biology (LCMB) are to determine the etiology of naturally occurring cancer and to elucidate mechanisms of transformation by carcinogenic agents. The ultimate aim of these investigations is to apply approaches successful in animal model systems to the identification of causative agents of human malignancies and to the prevention of cancer in man.

Many ongoing investigations within the laboratory derive from our research on retroviruses. These viruses are unique among animal viruses in their mode of transmission and the intimate association that has evolved between these agents and cells of a large number and wide variety of vertebrate species. The etiologic role of this virus group has been established for naturally occurring cancers of many species. Certain retroviruses, the so-called "replication-defective" transforming viruses, have arisen by a recombination with cellular genes or proto-oncogenes. Investigations within the laboratory have provided strong evidence that proto-oncogenes are frequent targets of genetic alterations that convert them to oncogenes and lead human cells along the pathways to malignancy. Today, much of our research is aimed at identifying genes that can be implicated as oncogenes in human cancer, their molecular mechanisms of activation, as well as the mode of action of their translational products. Our findings that proto-oncogenes can encode growth factors as well as subsequent discoveries that such genes can encode growth factor receptors have strongly implicated subversion of normal growth factor-activated pathways of cell proliferation in the neoplastic process. Thus, today a considerable portion of our research efforts concerns these important genes and the cascade of biochemical events involved in mitogenic signalling. The aim of these studies is the identification of new oncogenes, as well as strategies that may eventually be useful in blocking neoplastic progression.

The lentiviruses, a subfamily of the retroviruses, have been implicated as the causative agents of nonneoplastic diseases of certain animal species and of acquired immunodeficiency syndrome (AIDS) in man. Investigations in our laboratory utilize animal lentiviruses as models for treatment and prevention of AIDS.

Growth Factors

Platelet-derived Growth Factor/*sis*. Human platelet-derived growth factor (PDGF) is composed of two polypeptide chains, PDGF-A and PDGF-B, the human homolog of the v-*sis* oncogene. The discovery that the v-*sis* oncogene encodes a protein closely related in its predicted amino-acid sequence to a major component of human PDGF provided the first evidence that growth factor-mediated proliferative pathways played an important role in tumorigenesis. Available evidence indicates that the growth factor properties of the v-*sis* translational product are directly responsible for its transforming activity. Thus, it appears that v-*sis* expression constitutively activates a normal proliferative pathway, providing the affected cell with a constant growth stimulus which can contribute to malignant transformation.

A retrovirus containing the entire human PDGF-B gene was constructed in order to investigate the in vivo biological activity of its encoded growth factor. When this virus was introduced into newborn mice, it reproducibly generated fibrosarcomas at the site of inoculation. Evidence that the normal growth factor-coding sequence was unaltered derived from RNAase protection studies and immunoprecipitation analysis. Tumors were polyclonal but demonstrated clonal subpopulations. Moreover, tumor-derived cell lines rapidly became monoclonal upon growth in culture. These findings argue that PDGF-B overexpression can provide the initiating event for development of a clonal malignancy. Proviruses in each fibrosarcoma analyzed contained a deletion of 149 nucleotides downstream of the PDGF-B coding region. This deletion originated from an alternative or aberrant splice event which occurred within exon 7 of the PDGF-B gene and mimicked the v-*sis* oncogene. Thus, deletion of this region must confer a growth advantage to cells transformed by these retroviruses or be necessary for efficient retrovirus replication.

We investigated the regulation of PDGF-B mRNA expression during megakaryocytic differentiation of K562 cells. Induction by 12-O-tetradecanoylphorbol-13-acetate (TPA) led to a greater than 200-fold increase in PDGF-B transcript levels in these cells. Induction was dependent on protein synthesis and was not enhanced by cycloheximide exposure. In our initial investigation of the PDGF-B promoter, a minimal promoter region, which included sequences extending only 42 base pairs upstream of the TATA signal, was found to be as efficient as 4 kilobase pairs upstream of the TATA signal in driving expression of a reporter gene in uninduced K562 cells. We also functionally identified different regulatory sequence elements of the PDGF-B promoter in TPA-induced K562 cells. One region acted as a transcriptional silencer, while another region was shown to bind nuclear factors and was the target for *trans*-activation in normal and tumor cells. In one tumor cell line, which expressed high PDGF-B mRNA levels, the presence of the positive regulatory region resulted in a 30-fold increase in promoter activity. However, the ability of the minimal PDGF-B promoter to drive reporter gene expression in uninduced K562 cells and normal fibroblasts, which contained no detectable PDGF-B transcripts, implies the existence of other negative control mechanisms beyond the regulation of promoter activity.

The transforming activities of PDGF-A and PDGF-B genes were compared. The PDGF-A chain gene was markedly less efficient in inducing transformation than the PDGF-B gene under the influence of the same promoter. There are significant differences in the secretory and growth stimulating properties of the two chains. These properties appear to account for the much more potent transforming ability of the PDGF-B gene. These findings provide insights into biologic properties of a growth factor responsible for potent autocrine stimulation of abnormal cell proliferation.

By use of an appropriately engineered baculovirus expression vector, the v-*sis* protein was expressed in the insect cell line *Spodoptera fungiperida* (Sf9) at a level 50 to 100-fold greater than that observed with overexpression in mammalian cell transfectants. The *sis* protein produced by Sf9 cells underwent processing similar to that observed in mammalian cells including efficient disulfide-linked dimer formation. Moreover, the recombinant *sis* protein was capable of binding PDGF receptors and inducing DNA synthesis as efficiently as PDGF-B synthesized by mammalian cells. A significant fraction of *sis* protein was released from Sf9 cells, which made possible a one-step immunoaffinity purification to near homogeneity with a 40% recovery of biological activity. These results demonstrate that a protein whose normal processing requires both interchain disulfide bridge formation can be efficiently expressed in a biologically active form in insect cells using a baculovirus vector system.

bFGF. Basic fibroblast growth factor (bFGF) is a potent mitogen for a wide variety of cell types. Unlike most growth factors, the primary translation product for bFGF appears to lack a secretory signal peptide. To explore the normal mode of bFGF release, as well as to investigate the growth factor's oncogenic potential, expression vectors were created for a bFGF cDNA and for a chimeric molecule in which the bFGF coding sequence was linked to the human growth hormone signal peptide sequence. Transfection of NIH/3T3 cells with the bFGF cDNA vectors caused the synthesis of high levels of biologically active, cell-associated bFGF, but no evidence of transformation was detected. In contrast, the chimeric bFGF signal peptide expression vector induced foci of transformation at a very high frequency. The transformed cells grew in soft agar and were tumorigenic in nude mice. The majority of the immunoreactive bFGF species made by the transformed cells was found in the conditioned medium and appeared to be posttranslationally modified, indicating that the chimeric bFGF signal peptide molecule was processed through the secretory pathway. The secreted bFGF exhibited little mitogenic activity, suggesting that interaction of bFGF with its receptor likely occurs while the fusion protein is being processed along the secretory pathway.

TGFα. To investigate the role of transforming growth factor α (TGFα) in tumor development, we introduced the human TGFα (hTGFα) cDNA into cultured primary mouse epidermal cells or papilloma cells using a replication-defective retroviral vector and analyzed skin grafts constructed with such cells. Expression of the exogenous gene was confirmed by detection of hTGFa mRNA by northern RNA blot analysis, and the secreted hTGFα was measured by ELISA of culture supernatants. Tumor cells expressing hTGFa produced benign tumors (papillomas) which were 1.5- to 2-fold larger than tumors of parental cells when tested as skin grafts on nude mice. Grafts of normal cells that expressed hTGFa produced normal skin. When mixtures of parental tumor cells and normal mouse keratinocytes were grafted to nude mice, papilloma formation was suppressed and tumors that did form were small. Grafts of hTGFα-producing papilloma cells combined with either normal epidermal cells or hTGFα-producing epidermal cells yielded large tumors. Mixed grafts containing keratinocytes expressing hTGFα and parental papilloma cells also produced large tumors. While the tumor size was substantially increased by hTGFα expression, the tumors that developed in all groups were histologically benign and reached a stable size 4-5 weeks after grafting. These results indicate that expression of hTGFα by either tumor cells (autocrine) or adjoining normal cells (paracrine) can stimulate tumor growth, particularly when tumor growth is suppressed by normal tissue. However, expression of this growth factor did not appear to influence tumor progression directly.

KGF. A novel growth factor specific for epithelial cells was identified in conditioned medium of a human embryonic lung fibroblast cell line. The factor, provisionally termed keratinocyte growth factor (KGF) because of its predominant activity on this cell type, was purified to homogeneity by a combination of ultrafiltration, heparin-Sepharose affinity chromatography, and hydrophobic chromatography on a C4 reversed-phase HPLC column. KGF was both acid and heat labile and consisted of a single polypeptide chain of ~28 kiladaltons. Purified KGF was a potent mitogen for epithelial cells, capable of stimulating DNA synthesis in quiescent BALB/MK epidermal keratinocytes by >500-fold with activity detectable at 0.1 nM and maximal at 1.0 nM. Lack of mitogenic activity on either fibroblasts or endothelial cells indicated that KGF possessed a target cell specificity distinct from any previously characterized growth factor. Microsequencing revealed an amino-terminal sequence containing no significant homology to any known protein. The release of this growth factor by human embryonic fibroblasts raises the possibility

that KGF may play a role in mesenchymal stimulation of normal epithelial cell proliferation.

Growth Factor Receptors

A genomic sequence and cloned complementary DNA has been identified for a novel receptor-like gene of the PDGF receptor/colony stimulating factor type 1 (PDGF receptor/CSF1) receptor subfamily. The gene recognized a 6.4-kilobase transcript that was coexpressed in normal human tissues with the 5.3-kilobase PDGF receptor mRNA. Introduction of complementary DNA of the novel gene into COS-1 cells led to expression of proteins that were specifically detected with antiserum directed against a predicted peptide. When the new gene was transfected into COS-1 cells, a characteristic pattern of binding of the PDGF isoforms was observed which was different from the pattern observed with the known PDGF receptor. Tyrosine phosphorylation of the receptor in response to the PDGF isoforms was also different from the known receptor. The new PDGF receptor gene was localized to chromosome 4q11-4q12. The existence of genes encoding two PDGF receptors that interact in a distinct manner with three different PDGF isoforms likely confers considerable regulatory flexibility in the functional responses to PDGF.

*erb*B-2. Because of our interest in genes coding for growth factor receptors, we used the v-*erb*B gene to probe for related genes that might be candidates for receptor coding sequences. This led to the discovery of a novel epidermal growth factor (EGF) receptor-related gene, designated *erb*B-2. This gene was found amplified and/or overexpressed in a significant fraction of human mammary adenocarcinomas, and its direct oncogenic potential in vitro was demonstrated.

Compared with normal *erb*B-2 gp185, mutant *erb*B-2 proteins generated by mutations either in the transmembrane domain or by NH2-terminal deletion are able to transform NIH/3T3 cells at a 10- to 100-fold greater efficiency. Mutant proteins of both classes show increased tyrosine kinase activity, suggesting that an abnormal level of receptor-associated tyrosine kinase activity is a major determinant of *erb*B-2 oncogenic potential.

Through the use of a cDNA probe, the human *erb*B-2 gene was localized by *in situ* hybridization of normal human chromosomes at 17q11-q21. *In situ* hybridization of chromosomes derived from fibroblasts carrying a constitutional 15;17t(q22.3;q11.21) translocation showed that the *erb*B-2 gene was relocated on the rearranged chromosome 15. These results as well as grain localization on prophase chromosomes locate the *erb*B-2 gene at 17q12-q21.32. This localization may facilitate the search for human malignancies with chromosome changes involving the *erb*B-2 gene.

Our in vitro observations were paralleled by in vivo findings that a number of human mammary tumors and tumor cell lines display overexpression of the *erb*B-2 gene. In addition, we demonstrated that the levels at which the *erb*B-2 protein is capable of inducing an in vitro transformation are comparable to those detected in naturally occurring tumors exhibiting *erb*B-2 overexpression. Recently, an inverse correlation of *erb*B-2 gene amplification and disease-free survival has been reported in breast cancer patients, suggesting a predictive role of *erb*B-2 gene amplification in an aggressive disease course in human mammary neoplasia. Taken together, these observations establish a mechanistic basis for growth factor receptor gene amplification and overexpression as representing a causal driving force in the clonal evolution of a tumor cell rather than being an incidental consequence of tumorigenesis.

Membrane protein levels of *erb*B-2 and EGF receptor as well as gene aberrations affecting these proto-oncogenes in human mammary cancer were determined in primary and metastatic lesions. Among 57 patients, *erb*B-2 gene amplification was detected in 11 tumors (19%). In 10 of these patients where expression levels could be assayed, gene amplification was associated with a high level of *erb*B-2 protein. In contrast, EGF receptor gene amplification with overexpression of the protein product was observed in two tumors (4%). In addition, 14 out of 53 (26%) primary tumors exhibited moderately increased *erb*B-2 protein levels in the absence of gene amplification. Similar aberrations resulting in overexpression of EGF receptor protein without detectable gene amplification were associated with two tumors (4%) among 47 patients analyzed. In seven patients, expression level and gene copy numbers of *erb*B-2 or EGF receptor were similarly altered in the primary tumor and metastatic lesions derived from the same patient. Concordance of increased receptor gene expression in primary and metastatic lesions, combined with the observation that such alterations are detectable as early as stage I and II mammary tumors, suggests that overexpression of *erb*B proto-oncogene family members can develop early in breast cancer and is maintained during tumor progression. Comparison of *erb*B-2 overexpression with clinical disease parameters revealed a correlation of this alteration with inflammatory mammary carcinoma (P=0.042), implying an association of elevated *erb*B-2 protein levels with enhanced malignancy of the tumor cell in vivo.

Epidermal Growth Factor Receptor (EGFR). Alterations affecting the epidermal growth factor (EGF) transforming growth factor α (TGFα)-responsive mitogenic pathway are frequently detected in malignancies. In particular, the EGF receptor (EGFR) molecule has been found overexpressed in a number of human tumors, and TGFα is produced by a large array of tumor cells. Gene transfer experiments have previously demonstrated that expression of either TGFα or EGFR alone is not sufficient to induce the transformed phenotype in NIH/3T3 cells. We sought to investigate the biological effect of expression of TGFα and high levels of EGFR in this model system. We demonstrated that the gene for TGFα acts as a potent oncogene in NIH/3T3 cells overexpressing EGFR (NIH-EGFR, >106 EGFR). We further show that TGFα directly stimulates proliferation of the cell in which it is produced and provides evidence that the extracellular compartment of the transformed cell is the major site of interaction between TGFα and EGFR. Analysis of human tumor cell lines revealed a strong correlation between expression of TGFα and overexpression of EGFR. Moreover, high levels of EGF-independent tyrosine phosphorylation of the EGFR were detected both in NIH-EGFR expressing TGFα and in high EGFR and TGFα coexpressing human tumor cell lines. Thus, the two events instituting the EGFR/TGFα autocrine loop responsible for transformation in vitro may play a role in the development of some human malignancies.

Other Oncogenes

dbl. We isolated cDNA clones representing the human *dbl* proto-oncogene transcript. Nucleotide sequence analysis revealed an open reading frame encoding a predicted protein of 925 amino acids. Using peptide antisera directed against specific proto-*dbl* peptides, a 115-kd protein was detected in COS cells transfected with an expression vector containing the entire coding region of proto-*dbl*. This molecular weight is consistent with that predicted from the open reading frame. We have previously shown that the *dbl* oncogene was generated by substitution of the 5' portion of proto-*dbl* with an unrelated human sequence. In this study we show that this rearrangement resulted in the loss of the 497 amino-terminal codons of the *dbl* proto-oncogene. Under the influence of a strong promoter, proto-*dbl* could readily transform NIH/3T3 cells but its transforming activity was less than that of the *dbl* oncogene

driven by the same promoter. Proto-*dbl* expression is, therefore, sufficient to transform NIH/3T3 cells, but specific structural alterations of its coding region significantly enhance its transforming activity. No apparent similarity was detected between the predicted proto-*dbl* product and other known proto-oncogenes. However, a stretch of 300 amino acids within the N-terminal half of proto-*dbl* showed structural similarity to the intermediate filament vimentin. This region in proto-*dbl* contains a heptad repeat motif characteristic of an a-helical coiled-coil structure. Taken together, these findings indicate that the human proto-*dbl* represents a new class of cellular oncogenes that may be related to cytoskeletal elements of the cell.

The *dbl* oncogene was generated by rearrangements involving three discontinuous regions of the human genome. Analyses of panels of human x rodent somatic cell hybrids demonstrated that the *dbl* gene located on the X chromosome (just proximal or distal to bands q26-27.2) underwent recombination at its 5' and 3' ends with sequences derived from chromosomes 3 (p13q-ter) and 16 (p13-q22), respectively. *Dbl* was more precisely localized to chromosome Xq27-q28 by *in situ* hybridization. Another oncogene, *mcf*.2, was previously shown to contain sequences derived from Xq27 as well. Comparison of the restriction maps and nucleotide sequences of genomic and cDNA clones representing the chromosome X-specific sequences of *dbl* and *mcf*.2, taken together with their chromosomal localization, indicates that these oncogenes were derived from the same genetic locus.

fyn. The *src* gene is the prototype for a family of closely related genes whose products have protein-tyrosine kinase activity. We recently described another member of this family, designated *fyn*, whose cDNA was isolated from normal human fibroblasts. To examine the possible role of *fyn* as an oncogene, we investigated the effects of *fyn* overexpression on NIH/3T3 cells. Our findings demonstrate that normal *fyn* overexpression induces morphologic transformation and anchorage-independent growth. In addition, at relatively low frequency, *fyn* acquired properties of a dominant-acting oncogene capable of inducing the fully tumorigenic phenotype. Genetic changes associated with the conversion of normal *fyn* cDNA into a transforming gene with high focus-forming activity were localized to the carboxyl-terminal region of its translational product.

ras. The very high frequency of detection of *ras* oncogenes in human tumors strongly suggests that these genes play a role in the processes leading to the formation of a tumor. It has been possible to ascertain that in patients whose tumor cells possess an activated *ras* oncogene, the activating lesion is not found in the *ras* alleles of normal cells. Furthermore, analysis of biologically cloned tumor cells from such a patient showed that all tumor cells contained the activated allele. These findings establish that mutations that activate *ras* oncogenes occur somatically and that these events are powerfully selected for within the tumor. All of the above findings, taken together, imply that the activation of these oncogenes is part of the cause rather than a result of the neoplastic process.

Activation of the cellular oncogene c-N-*ras* has been frequently observed in DNA from leukemic cells in acute myeloid leukemia (AML). *Ras* gene activation sufficient to mediate in vitro transformation and rodent tumorigenesis usually results from point mutations and amino acid substitutions in the 12th or 61st codons. In AML and the related myelodysplastic syndromes, amino acid substitution at the 13th codon has been observed. An activated c-N-*ras* gene from a 45 year old patient with AML was isolated by transfection analysis and subjected to molecular cloning and sequence analysis. A point mutation of the 12th codon (GGT to GAT) resulting in aspartic acid substitution for glycine

was observed. In other neoplasms such as colon cancer, specific *ras* mutations occur predominantly (e.g., K-*ras*, codon 12). This predominance has been of demonstrable value in analyzing large cohorts for *ras* activation with techniques that are rapid and economical, such as oligonucleotide hybridization. It had previously been thought that such a predominance for activation of c-N-*ras* at codon 13 existed in AML; however, this study in concert with others underscores the importance of 12th codon c-N-*ras* mutations, along with 13th and 61st codon mutations in the molecular pathogenesis of AML. Guanylate to adenylate transition mutations are commonly observed in AML and may provide insight into potential environmental leukemogens. Addressing all commonly prevalent *ras* activation mutations bears impact in the future design of molecular surveys of the role of *ras* activation in leukemogenesis.

Two H-*ras* oncogenes were detected by NIH/3T3 transfection assay out of 16 primary kidney tumors, 15 renal cell carcinomas and one transitional cell carcinoma. Analysis of *ras M*, 21,000 protein suggested single point mutations within codon 12 and 61 in each case. The restriction endonuclease analysis of the H-*ras* gene at codon 12 confirmed this in one of them, and the remaining 15 tumors did not have a mutation at this site. DNAs from the noncancerous portions of the kidney with codon 12 mutated tumor, but not leukocytes from the same patient, showed an abnormal resistance to the endonucleases *Msp*I and *Hpa*II, suggesting the presence of a codon 12-mutated H-*ras* gene in the noncancerous cells. No amplification of *ras* genes was detected in the 16 tumors analyzed. In one of eight tumors from patients heterozygous for H-*ras*-related *Bam*HI restriction fragments, one allele was lost in the tumor but not in the noncancerous portion of the same kidney. Although cytogenetic studies have previously suggested nonrandom involvement of the c-*raf*-1 gene in renal cell carcinomas, no abnormality in the size or amount of the *raf* transcript was detected in the 15 renal cell carcinomas. Our results indicate that the genetic lesions affecting *ras* genes do occur in human renal cell carcinomas and probably serve as one of several multisteps in the carcinogenic process.

The human osteosarcoma cell line Te85 clone F-5 is not tumorigenic in vivo. Its transformation with Kirsten murine sarcoma virus (KiMSV)(KHOS) confers full malignant properties and stable nontumorigenic revertants of this KHOS cell line have been obtained. We showed that integration and expression of a single copy of the Ki-MSV proviral DNA, which is totally lost in the HOS 240S revertant, is responsible for the acquisition of tumorigenicity. Cytogenetic analysis and the absence of a residual LTR copy in the revertant cellular genome suggest that the loss of KiMSV provirus is caused either by chromosomal segregation or by recombination not involving the LTR. In addition, analysis of the expression of *ras* proteins revealed no changes in the pattern of c-*ras* products and the expression of v-*ras* only in the KHOS cells. All these data suggest that Te85 and HOS 240S cell lines could represent a human alternative recipient system to rodent cells in studies with oncogenes.

Many human bronchial adenocarcinomas have been shown to contain an activated Ki-*ras* oncogene. To test the hypothesis that activated Ki-*ras* may be causally related to human bronchial carcinogenesis, the v-Ki-*ras* oncogene was transferred into an established human bronchial epithelial cell line, BEAS-2B, by infection with Ki-MSV or by transfection with a plasmid containing the transforming region of Ki-MSV. These cells formed poorly differentiated adenocarcinomas in athymic nude mice. Cell lines established from these tumors expressed the v-Ki-*ras* p21 protein and were highly tumorigenic. Whereas serum or transforming growth factor β_1 induced the BEAS-2B cells at clonal density to undergo growth arrest and squamous differentiation, BEAS-2B

cells containing activated *ras* genes were unaffected by transforming growth factor b1 and were mitogenically stimulated by serum.

Epithelial Cell Models for Normal Growth, Differentiation and Malignancy

BALB/MK epidermal keratinocytes require EGF for growth in serum-containing medium and terminally differentiate in response to high Ca^{++} concentration. Several oncogenic retroviruses have been shown to relieve the EGF requirements and to block calcium-induced terminal differentiation. We developed a chemically defined medium to investigate the minimum growth factor requirements for BALB/MK cells, as well as how such requirements might be altered by retroviral oncogenes. In this medium insulin, apparently acting as IGF-1 and EGF, supported cell growth in a manner comparable to serum and EGF. Acidic as well as basic fibroblast growth factors (FGF) substituted for EGF but not insulin in supporting BALB/MK proliferation. Infection with retroviruses containing v-*ras* oncogenes (v-H-*ras*, v-K-*ras*), oncogenes derived from growth factor receptors (v-*erb*B, v-*fms*) or the v-*mos* oncogene permitted growth in defined medium containing insulin but lacking EGF. The v-*fgr* oncogene, a member of the *src* subfamily, was unique in conferring independence insulin and EGF. Our findings establish the applicability of this system for biologic assay of epithelial cell growth factors as well as identification of specific growth factor requirements that can be altered or complemented by the actions of specific oncogenes.

Using a defined medium culture system, we investigated the role of physiological concentrations of EGF on phosphoinositide metabolism in these cells. The results show that EGF rapidly activates phospholipase-C-mediated phosphoinositide metabolism resulting in the generation of the second messengers, inositol 1,4,5-trisphosphate and diacylglycerol. These metabolites control intracellular Ca^{++} levels and activate protein kinase C, respectively. Protein kinase C activation in response to EGF was evidenced by the phosphorylation of the acidic 80 kilodalton endogenous protein substrate (p80) specific for this kinase. In contrast, insulin, which acts in concert with EGF to cause BALB/MK cell proliferation, had no effect on phosphoinositide metabolism nor led to any additional stimulation when added in combination with EGF. Taken together, our results show that rapid alterations in phosphoinositide metabolism and protein kinase C activation are associated with the normal mitogenic response of keratinocytes to EGF.

While it is known that BALB/MK keratinocytes require EGF for proliferation and terminally differentiate in response to elevated extracellular Ca^{++} concentrations, the molecular pathways controlling cell differentiation in this system have yet to be established. We showed that a dramatic and sustained activation of phosphoinositide metabolism is produced upon addition of Ca^{++} to BALB/MK cultures. The pattern of inositol trisphosphate isomers released in response to Ca^{++} challenge appeared to be atypical. Inositol 1,3,4-trisphosphate release was observed by 30 seconds and was produced earlier than any alteration in inositol 1,4,5-trisphosphate levels. Concomitant with the liberation of inositol phosphates, an increased production of diacylglycerol was observed. Despite a threefold increase in diacylglycerol levels detected even at 12 hours after Ca^{++} addition, no evidence of functional activation or downregulation of protein kinase C was found. This was established by measuring p80 phosphorylation, EGF binding and protein kinase C levels by immunoblotting. Analysis of the diacylglycerol generated following Ca^{++} addition to BALB/MK cells revealed that a significant proportion of that lipid is an alkyl-ether-glyceride molecular species. Therefore, it is possible that this diacylglycerol molecular species may play

a role in the Ca^{++}-induced differentiation program of BALB/MK cells through mechanisms other than stimulation of classical protein kinase C.

V-oncogenes in human epithelial cell transformation. The development of tissue culture systems for propagation of human epithelial cells has aided the investigation of events that lead epithelial cells to become neoplastic. Nontumorigenic human epidermal keratinocytes, immortalized by adenovirus 12 (Ad12)-SV40 virus or pSv3-neo, were transformed by a variety of retroviruses containing *bas*, H-*ras*, *fes*, *fms*, *erb*B and *src* oncogenes. Such transformants showed morphological alterations and induced carcinomas when transplanted into nude mice. These findings demonstrate the malignant transformation of human primary epithelial cells in culture by the combined action of AD12-SV40 virus and retroviral oncogenes and support a multistep process for neoplastic conversion. This in vitro system may be useful in studying the interaction of a variety of retroviral oncogenes and human epithelial cells.

Hematopoietic Cell/Oncogene Studies

We have demonstrated the derivation of clonally related undifferentiated progenitors, pre-B cells, B cells and macrophages following in vitro infection of murine bone marrow cells with combined v-*raf*/v-*myc* oncogenes. The heterogeneous phenotypes obtained were not due to differences in target cell distribution based on LTR regulatory sequence differences, as the vectors in this analysis were constructed with identical LTR sequences. The demonstration of clonality strongly suggests that the target of v-*raf*/v-*myc*-induced transformation is common to both B and myeloid lineages.

We have found that differences in steady state c-*myb* mRNA levels between murine B lymphoid tumors are maintained primarily by a block to transcription elongation rather than at the level of transcription initiation. Similarly, the differential expression of c-*myc* mRNA has been reported to be regulated, at least in part, by a block to transcription elongation. As expression of both c-*myc* and c-*myb* is associated with cell growth and proliferation regulation of transcription at the level of elongation may provide a more rapid and sensitive mechanism to rapidly increase or decrease steady state mRNA in response to external signals than transcription initiation. Thus, it is of interest that expression of both c-*myc* and c-*myb* mRNA increases during progression from the G0 through the G1 stage of the cell cycle. By keeping these genes in a transcriptionally active state, simple removal or alteration of a block to elongation would allow rapid increases in steady state mRNA levels.

To define the action of the retroviral *src* gene on hematopoietic stem cells, B6D2F1 mouse long-term marrow cultures were infected at initiation with Moloney-MuLV pseudotypes of *src*-recombinant retroviruses with the *src* gene inserted in the *env* region of an amphotropic MuLV (*src*-ampho), or in the *gag* region of Moloney-MuLV (*src*-Mo). Other cultures were infected with Friend spleen focus-forming virus polycythemia-inducing strain (SFFVp), Moloney-MuLV, amphotropic-MuLV or were uninfected controls. Cultures infected with *src*-ampho, *src*-Moloney, or SFFVp demonstrated a significant increase in cumulative nonadherent cell and CFU-GEMM production. There was prolonged self-renewal over seven serial transfers of individual *src* virus-negative CFU-GEMM from *src* virus-infected cultures, similar to that with permanent interleukin-3 (IL-3)-dependent cell line B6SUtA. In contrast, MuLV-infected or control uninfected cultures produced fewer cells and self-renewal of CFU-GEMM did not exceed three generations. IL-3-dependent clonal hematopoietic progenitor cell lines derived from each culture group formed no detectable tumors in vivo. However,

each released the original helper and/or transforming virus. In contrast, v-*src* gene expression by transfection of IL-3-dependent cell line 32D cl 3 produced factor-independent growth and tumor formation in vivo. Adherent cell lines, derived from *src*-ampho-infected cultures released *src* virus and formed fibrosarcomas in vivo. Thus, *src* recombinant virus expression is regulated distinctly by multilineage hematopoietic stem cells, established IL-3-dependent cell lines and adherent cells from long-term marrow cultures.

The effects of murine recombinant IL-3 and murine granulocyte-macrophage colony stimulating factor (GM-CSF) on the radiation biology of clonal hematopoietic progenitor cell lines were evaluated. It was demonstrated that some hematopoietic progenitor cell lines are induced by GM-CSF to grow after irradiation at low dose rate similar to the growth of clonal malignant cell lines. The data may have significant implications for the radiation biology of normal hematopoietic progenitor cells in two circumstances: (a) selective survival of GM-CSF responsive cells after total body irradiation, and (b) selective survival of some hematopoietic progenitors in vivo during clinical recombinant GM-CSF infusion.

DNA Repair and Cancer Susceptibility

Skin fibroblasts or peripheral blood lymphocytes from individuals with genetic disorders predisposing to cancer or with familial cancer show a higher than normal incidence of chromatid breaks and gaps when irradiated during G_2 phase of the cell cycle. The incidence is also higher in human tumor cells and cells transformed in culture than in normal controls. This enhanced G_2 chromatid radiosensitivity is thus associated with both genetic susceptibility to cancer and neoplastic transformation. It is observed only in cells harvested at least 1.5 hours after irradiation and appears to result from a deficiency(ies) in DNA repair during G_2 phase. This deficiency has a genetic basis, behaving as a recessive trait. Furthermore, G_2 chromatid radiosensitivity provides a means for identifying individuals with genetic susceptibility to cancer.

The frequencies of chromatid breaks and gaps in metaphase cells fixed two hours after G_2 phase x-irradiation (1 Gy) were in almost all cases at least two- to threefold higher in skin fibroblasts from individuals with genetic conditions predisposing to cancer than in comparable cells from clinically normal controls. Previously, we reported this response in all cancer-prone genetic disorders tested including ataxia telangiectasia, Bloom's syndrome, Fanconi's anemia, xeroderma pigmentosum (XP), familial polyposis, Gardner's syndrome, hereditary malignant melanoma, dysplastic nevus syndrome and cancer family members. One exception was XP-A. In this report we add information on skin fibroblasts from retinoblastoma, Wilms' tumor and XP-C patients, 13 clinically normal controls and six cell lines from fetal or infant cells. Factors affecting the response are identified and include pH, temperature, cell density, culture medium or serum, microbial contamination and visible light exposure (effective wavelength 405 nm). Because of experimental variability, known normal controls should be used in each group of assays. With adequate control of the above factors, this response could provide the basis of a test for detecting individuals carrying genes that predispose to a high risk of cancer.

Early passage skin fibroblasts from different inbred and congenic strains of mice were x-irradiated (1 Gy) and the number of chromatid breaks was determined at 2.0 hours after irradiation. The cells from DBA/2N, C3H/HeN, STS/A, C57BL/6N, BALB/cJ, and AKR/N had 25 to 42 chromatid breaks per 100 metaphase cells (efficient repair phenotype). NZB/NJ had >78 and BALB/cAn had

87 to 110 chromatid breaks per 100 cells (inefficient repair phenotype). Differences between BALB/cAn and BALB/c.DBA/2 congenic strains which carry less than 1% of the DBA/2 genome indicate that two genes, one on chromosome 1 linked to *bc1-2-Pep-3* and the other on chromosome 4 closely linked to *Fv-1*, affect the efficiency with which the cells repair radiation-induced chromatin damage.

The C.D2-*Fv-1* (N19) mouse carries both a gene on the distal end of chr-4 that determines partial resistance to plasmacytoma development and a gene for efficient chromatin repair. This suggests the possibility that both phenotypes are controlled by the same gene. If so, one of the genes that determines the S phenotype of BALB/cAn mice may operate by increasing the chances for the development of DNA damage that might predispose to the formation of chromosomal translocations. This must be regarded as speculative for a number of reasons. First the distal end of chr-4 contains approximately 10cM or more of DBA/2 chromatin in C.D2-*Fv-1* mice. BALB/cAn and DBA/2N then could differ in several different allelomorphic genes in this region of the chromosome. Second we have not tested the C-D2-*Fv-1* N19 mouse for partial resistance to plasmacytoma induction. The C-D2-*Fv-1* N10 mouse did express partial resistance but during continued backcrossing the C.D2 *Fv-1* (N19) mouse no longer had the DBA/2 allele of Mmtv-13. Thus, it is possible that the plasmacytoma R gene could be more closely linked to the Mmtv-13 locus and hence is no longer present in the N19 mouse. The working hypothesis is that the inefficient repair phenotype is associated with an inability to correct double-stranded DNA breaks. The assay system employed specifically examines chromatid breaks that are a result of double-stranded DNA breaks incurred during G_2 phase of the cell cycle. While the assay system only determines the capability for repair during G_2, similar lesions that develop in other stages of the cell cycle may also be inefficiently repaired. Chromatid breaks that are unrepaired in G_2 may lead to lethality during subsequent mitoses if significant losses of DNA are sustained. If, however, double-stranded breaks occur in different chromosomes that happen to be neighbors during G_1 to S phase, then other factors may promote illegitimate recombinations such as those seen in reciprocal translocations. The primary effect of the inefficient *Rep* allele may be to delay normal repair processes and permit the formation of illegitimate recombination. The data suggests that there may be multiple genes in the mouse that affect the efficiency of DNA repair and susceptibility to pristane-induced plasmacytoma formation. We have a C.D2 congenic mouse that coincidently has the phenotype of partial resistance to plasmacytoma induction and efficient repair of x-ray-induced chromatid breaks. Further analysis will be required to determine if these are controlled by the same or different genes.

Virus Studies

We have developed immortalized epithelial cystic fibrosis (CF) cell lines by infecting cultured nasal polyp cells with an AD12-SV40 hybrid virus. The cell lines obtained were epithelial in nature as shown by cytokeratin production and morphology, although cytokeratins 4 and 13 typical of primary nasal polyp cells were produced at a much reduced rate. Ussing chamber experiments showed that the precrisis CF cell line NCF3 was able to perform transcellular chloride transport when activated by agents which elevate intracellular calcium. cAMP agonists had no effect on chloride flux in NCF3 as expected for CF cells. The apical chloride channels found with the patch clamp technique in NCF3 and in the postcrisis cell line NCF3A had a conductance similar to that of chloride channels found earlier in normal and CF epithelial cells. The channels showed a delay in the onset of activity in off-cell patches and were not activated by increased cAMP levels in the cell. This indicates that

immortalized CF epithelial cells will provide a useful model for the study of cystic fibrosis.

Sjogren's syndrome (SS) is an autoimmune disorder characterized by lymphocytic infiltration of salivary and lacrimal glands. Other investigators have demonstrated elevated Epstein-Barr virus (EBV) in the salivary glands of SS patients and suggested that EBV might play a role in its pathogenesis. In order to further study the relationship of EBV or the human herpesvirus 6 (HHV-6) to Sjogren's syndrome, epithelial cell cultures were established from SS salivary gland biopsies and normal individuals in a serum-free medium and cell lines were established by introducing the transforming region of SV40 DNA.

To aid in characterizing adenosine receptors in renal cells, primary cultures of rabbit corticol collecting tubule (RCCT) cells were infected with AD12-SV40, resulting in a continuous cell line. The cells, designated RCCT-28A, retained their epithelial morphology and reacted with a monoclonal antibody specific for rabbit collecting tubule. Adenosine 3',5'-cyclic monophosphate (cAMP) accumulation was stimulated by vasopressin (AVP), isoproterenol, prostaglandin E2 (PGE2), calcitonin, parathyroid hormone and a potent adenosine A_1- and A_2-receptor agonist, 5'-N-ethylcarboxamidoadenosine (NECA). A more selective adenosine A_1-receptor agonist, N^6-cyclohexyl adenosine (CHA) inhibited basal and AVP-stimulated c-AMP accumulation. Cytosolic-free calcium was transiently elevated by bradykinin, PGE_2, NECA and CHA. To examine the mechanism by which adenosine analogues increased intracellular-free calcium, phosphoinositide (PI) turnover was assessed in the 28A cells after labeling with myo-[^{3}H]inositol. NECA and CHA increased [^{3}H]inositol phosphate formation with an approximate half-maximal effective concentration of 0.1mM for both analogues. The increase in PI turnover was blocked by the selective adenosine A_1-receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine and pretreatment of the 28A cells with pertussis toxin. Results suggest that adenosine analogues increase cytosolic-free calcium by stimulating PI turnover.

Normal human bronchial epithelial cells were infected with SV40 virus or an AD12-SV40 hybrid virus, or transfected via strontium phosphate coprecipitation with plasmids containing the SV40 early region genes. Colonies of morphologically altered cells were isolated and cultured; these cells had extended culture life spans compared to normal human bronchial cells. All cultures eventually underwent senescence, with the exception of one which appears to have unlimited proliferative potential. Colonies arising after viral infection were screened for virus production by cocultivation with Vero cells; only viral nonproducer cultures were analyzed further. The cells retained electron microscopic features of epithelial cells, and keratin and SV40 T antigen were detected by indirect immunofluorescence. All of the cultures were aneuploid with karyotypic abnormalities characteristic of SV40-transformed cells. These cells should be useful for studies of multistage bronchial epithelial carcinogenesis.

Lentivirus studies. A full length molecular clone of the equine infectious anemia virus (EIAV) has been obtained. This virus, although unrelated to the DNA of uninfected horses and other species tested, is genetically related to both the retroviruses of acquired immune immunodeficiency syndrome (AIDS) and ovine and caprine lentiviruses in the *gag* and polymerase genes. These observations suggest that these viruses have evolved from a common progenitor and are evolutionarily closely related. The nucleotide sequence of the integrated form of the genome of EIAV has been determined. The molecular analysis of EIAV with respect to the requirements needed to develop a vaccine

will not only contribute to control an important pathogen of horses, but also will represent an important model for the development of vaccines to human retroviruses.

In order to provide more sensitive and convenient methods for the detection of EIAV, we developed an enzyme-linked immunosorbent assay (ELISA) employing the EIAV *gag* precursor ($Pr55^{gag}$) produced by using recombinant DNA techniques. The antigenic reactivity of the recombinant EIAV $Pr55^{gag}$ was found to be equivalent to that of the virion $p24^{gag}$ and elicited high titered antiserum in rabbits. When a large number of horse sera were analyzed for the presence of antibodies to EIAV by using this ELISA, a radioimmunoassay (RIA) for EIAV $p15^{gag}$, or the standard agar gel immunodiffusion test, there was 98.7% concordance among the assays. By using the ELISA it was possible to specifically detect antibodies earlier after experimental infection of horses with EIAV than the other two tests. A competition ELISA developed to detect EIAV *gag* antigens was found to be approximately 15 times more sensitive than the RIA for EIAV $p15^{gag}$. Antigens of other animal lentiviruses as well as those of prototype oncovirus failed to compete in this assay.

We analyzed the EIAV long terminal repeat (LTR) for sequences that influence its promoter activity and ability to be *trans*-activated by the EIAV *tat* gene product. The effects of LTR sequences derived from another animal lentivirus, caprine arthritis and encephalitis virus (CAEV), on the activity of the EIAV LTR were also assessed. A series of LTR deletion mutants and recombinants between LTR and SV40 regulatory sequences were used for these studies. We were able to identify the EIAV promoter region and showed that sequences within the U3 region significantly inhibited LTR-directed transcription. However, when placed in a heterologous context (SV40 promoter), these same U3 sequences functioned as an enhancer. By using the same approach, the CAEV LTR was also found to contain an enhancer within its U3 region. *Trans*-activation of the EIAV LTR was found to depend upon sequences downstream of the transcription initiation site and also within U3. Deletion mutagenesis experiments showed that the major downstream element was present in a 46-nucleotide stretch (+4 to +50). An SV40 promoter construct containing these sequences could be *trans*-activated in cells expressing the EIAV *tat* gene product.

Lymphoproliferative disease virus of turkeys. The lymphoproliferative disease virus of turkeys was molecularly cloned, structurally mapped and shown to represent a distinct class of retroviruses evolutionarily related to the avian leukemia-sarcoma group. The cloned provirus did not contain any known oncogene or other cellular-derived sequences and was established as a replication-competent oncogenic entity, inducing the disease in the absence of any associated transforming counterpart.

Herpesvirus saimiri. We studied the replication of *Herpesvirus saimiri* (HVS), strain H, in human cells as part of an effort to assess the risk of humans to this transforming herpesvirus. Earlier studies on the replication of HVS in human cells were carried out before the development of immunofluorescence and biochemical techniques which would have been useful in characterizing the interaction of the virus and host cells. In these studies, all human cells expressed HVS early antigens as detected by immunofluorescence and immunoprecipitation. Analysis of late antigens present in infected human cells using polyvalent sera and monoclonal antibodies indicated that many, but not all, were not synthesized at detectable levels. This imbalanced synthesis of late antigens and the low level of several major virion proteins were sufficient to explain the extremely low titers of HVS observed in cultures of human cells.

Human herpesvirus-6 (HHV-6). The role of HHV-6 as a primary etiological agent or as a cofactor in human disease was investigated. Approximately 12% of heterophile-negative infectious mononucleosis (IM) cases are caused by HHV-6. This was evidenced by the detection of IgM antibody to the HHV-6 virus capsid antigen (VCA) in 25 of 204 cases examined. The contributory role of HHV-6 in chronic fatigue syndrome (CFS) patients was demonstrated. Fifty-one percent of the CFS patients studied had elevated antibody to HHV-6 VCA as compared to age- and sex-matched healthy donors. Active HHV-6 infection was detected in the peripheral lymphocytes of 9/12 patients (4-30% antigen-positive cells), employing HHV-6 monoclonal antibody. Sera from one of 11 (<1%) normal donors contained HHV-6 VCA-positive cells. These data were supported by *in situ* studies using an HHV-6 DNA probe. Other diseases in which HHV-6 is implicated are Sjogren's syndrome, sarcoidosis, thyroiditis, Hodgkin's disease, B-cell lymphomas and acquired immunodeficiency disease syndrome (AIDS). Dual infection with HHV-6 and human immunodeficiency virus (HIV)-1 or HIV-2 of CD_4+ cells (fresh or cell lines) showed enhanced killing, a significant increase in HIV-1 reverse transcriptase activity, and the simultaneous presence of both viruses in the same cells, suggesting that HHV-6 may contribute directly or indirectly to the depletion of CD_4+ T-cells in AIDS.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP04930-18 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biology of Natural and Induced Neoplasia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. Arnstein	Veterinary Director	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. S. Rhim	Research Microbiologist	LCMB	NCI
	J. S. Pierce	Research Microbiologist	LCMB	NCI
	A. Eva	Visiting Scientist	LCMB	NCI
	D. Ron	Visiting Associate	LCMB	NCI
	M. Kraus	Visiting Scientist	LCMB	NCI
	S. B. Blam	NRSA Fellow	LCMB	NCI
	M. Pech	Visiting Scientist	LCMB	NCI

COOPERATING UNITS *(if any)*

State of California Department of Health Services, Berkeley, CA (J. Riggs and R. Emmons); Children's Hospital Medical Center, Oakland, CA (K. Walen)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.0	1.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to correlate in vitro effects of oncogene activity on cellular and viral biology with in vivo tumorigenesis. The oncogene erbB-2 was proven persistant in lines obtained by grafting or tissue culture of virally induced murine tumors. This oncogene also proved capable of directly transforming benign human breast epithelium into carcinomogenic sublines.

The oncogene bfgf transformed benign murine fibroblasts to sarcomagenic lines. When integrated into type C helper virus vectors, it consistently induced sarcomas following newborn inoculation of mice. Previously benign human keratinocytes were proven carcinomogenic following transfection with ras genes; x-irradiation also produced neoplastic transformation of keratinocytes. Benign murine hematopoietic line 32D became lymphomogenic following transfection with several oncogenes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. Arnstein	Veterinary Director	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. S. Rhim	Research Microbiologist	LCMB	NCI
J. H. Pierce	Research Microbiologist	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
M. H. Kraus	Visiting Scientist	LCMB	NCI
D. Ron	Visiting Associate	LCMB	NCI
S. B. Blam	NRSA Fellow	LCMB	NCI
M. Pech	Visiting Scientist	LCMB	NCI

Objectives:

1. Characterize selected oncogenes of particular interest to LCMB staff, with special emphasis on in vivo manifestation of neoplasia and parallel effects in vitro. The ultimate goal is to determine the full potential of pathogenic expression (or lack of pathogenicity) for each putative oncogene.

2. Characterize neoplastic transformation in selected tissue culture systems and correlate morphologic transformation with neoplastic transplantability in nude athymic mice.

3. Test spontaneous mammalian neoplasms, especially human, for xenograft malignancy and explore correlations with oncogene expression in the original tissue.

Methods Employed:

Specified oncogenes selected by other LCMB investigators and incorporated into viral vectors are tested for pathogenicity (particularly carcinogenicity) by inoculation into normal (euthymic) and immunodeficient (athymic nude) siblings by several routes (intraperitoneal, subcutaneous, intramuscular, intracerebral), usually at birth. The same oncogenes integrated into cellular genomes are tested by transplantation into athymic nude adult hosts. The hosts chosen include three levels of immunodeficiency: (a) T-cell deficiency, (b) T- + B-cell deficiency, (c) T- + B- + LAK-cell deficiency.

Special studies on oncogenic transformation of primate cells and neoplastic behavior of spontaneously expressed oncogenes in human malignancies (in collaboration with Drs. Aaronson, Project No. Z01CP04940-22 LCMB; Rhim,

Project No. Z01CP05060-11 LCMB; and Kraus, Project No. Z01CP05366-06 LCMB) utilize similar techniques.

Major Findings:

Continuing studies on the *erb*B-2 (*mac*) oncogene, in collaboration with Dr. J. Pierce, demonstrated persistence of specific activity in delayed vascular and cardiac tumors induced by infection with virus containing this sequence. Original cultures of these tumors as well as subsequent serial grafts in nude mice exhibited typical tumor formation, production of the oncogene protein

(related to epidermal growth factor receptor, EGFR) and release of transforming viruses. Most recently, *erb*B-2 pseudotype oncovirus has repeatedly produced malignant transformation of a chemically immortalized but otherwise normal and completely benign human breast line. The transformed sublines exhibit altered morphology, chronic production of *erb*B-2 transforming virus and induction of epidermoid carcinomas when grafted on nu/nu mice. Outgrowths from these tumors have been confirmed karyologically as human, bearing the appropriate clonal chromosomal markers.

The oncogene *bfgf* (basic fibroblast growth factor) was studied in collaboration with Dr. S. Blam. It proved to be a transforming agent for murine fibroblasts in vitro. Lines established from transformed cultures, when grafted, induced highly malignant sarcomas in nu/nu mice. In addition, pseudotype viruses containing the *bfgf* gene proved consistently sarcomagenic when inoculated into newborn mice; the tumors occurred in characteristic fashion in the sternal area at two to four months of age.

Studies on immortalized human keratinocytes, as adjuncts to Dr. J. Rhim's project, continue to demonstrate the susceptibility of this epithelium to malignant transformation by oncogenes; recently it has been shown that this step can be accomplished by DNA transfection of the oncogene *ras* (as well as by viral infection reported previously). Keratinocytes transfected by *ras* were subsequently grafted on nu/nu mice and produced epidermoid carcinomas. Similar malignant transformations were obtained with 200-400 rads of x-irradiation in this system.

In another model, benign interleukin 3-dependent hematopoietic cells (32D) were converted to graftable lymphoma lines by transfection with the oncogenes *erb*B, *sis*, platelet-derived growth factor receptor (PDGFR), EGFR and *erb*B-2.

Publications:

Pech M, Gazit A, Arnstein P, Aaronson SA. Generation of fibrosarcomas *in vivo* by a retrovirus which expresses the normal PDGF-B chain and mimics the alternative splice pattern of the v-*sis* oncogene. Proc Natl Acad Sci USA (In Press)

Rhim JS, Kawakami T, Pierce J, Sanford K, Arnstein P. Cooperation of v-oncogenes in human epithelial cell transformation. Leukemia 1988;12:151S-9S.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04940-22 LCMB

PERIOD COVERED: October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Viruses and Transforming Genes in Experimental Oncogenesis and Human Cancer

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.0	1.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goals of this project are to elucidate the mechanisms of action of tumor viruses and to determine the cellular alterations responsible for naturally occurring malignancies. Topics of present interest include: (1) transforming genes of retroviruses and cancer cells, (2) growth factor signalling pathways, (3) the molecular biology of retrovirus replication and transformation, and (4) the application of knowledge gained from these studies to the search for the causes and mechanisms involved in human neoplastic transformation.

Some of the highlights of the past year include: (1) demonstration that the expression of the normal PDGF-B coding sequence in cells possessing the cognate receptors can be the initial step in malignancy; (2) identification and purification of a novel human epithelial cell-specific growth factor, designated keratinocyte growth factor (KGF); (3) isolation of the cDNA of a new PDGF receptor and demonstration that its ligand binding and properties are distinct from those of a previously cloned PDGF receptor; (4) elucidation of the mechanism activating the epidermal growth factor (EGF) receptor-related gene, erbB-2, that is frequently amplified and/or overexpressed in human mammary carcinomas; (5) demonstration of conditions that activate the malignant potential of bFGF and TGFα; (6) isolation of the normal coding sequence of the dbl proto-oncogene and demonstration of its transforming potential when overexpressed in NIH/3T3 cells; and (7) demonstration of the major growth factor signalling pathway subverted by many oncogenes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
J. H. Pierce	Research Microbiologist	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
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O. Segatto	Visiting Associate	LCMB	NCI
T. Fleming	Guest Researcher	LCMB	NCI
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J. Moscat	Guest Researcher	LCMB	NCI

Objectives:

1. To study the mechanisms of action of RNA tumor viruses and transforming genes.

2. To apply knowledge gained from experimental systems to the search for etiologic agents and mechanisms involved in neoplastic transformation of human cells.

Methods Employed:

Standard and developmental techniques in virology, cell biology, immunology and molecular biology.

Major Findings:

I. Growth Factors

Platelet-derived Growth Factor/*sis*. Human platelet-derived growth factor (PDGF) is composed of two polypeptide chains, PDGF-A and PDGF-B, the human homolog of the v-*sis* oncogene. The discovery that the v-*sis* oncogene encodes a protein closely related in its predicted amino-acid sequence to a major component of human PDGF provided the first evidence that growth factor-mediated proliferative pathways played an important role in tumorigenesis. Available evidence indicates that the growth factor properties of the v-*sis* translational product are directly responsible for its transforming activity. Thus, it appears that v-*sis* expression constitutively activates a normal proliferative pathway, providing the affected cell with a constant growth stimulus which can contribute to malignant transformation.

A retrovirus containing the entire human PDGF-B gene was constructed in order to investigate the in vivo biological activity of its encoded growth factor. When this virus was introduced into newborn mice, it reproducibly generated fibrosarcomas at the site of inoculation. Evidence that the normal growth factor-coding sequence was unaltered derived from RNAase protection studies and immunoprecipitation analysis. Tumors were polyclonal but demonstrated clonal subpopulations. Moreover, tumor-derived cell lines rapidly became monoclonal upon growth in culture. These findings argue that PDGF-B overexpression can provide the initiating event for development of a clonal malignancy. Proviruses in each fibrosarcoma analyzed contained a deletion of 149 nucleotides downstream of the PDGF-B coding region. This deletion originated from an alternative or aberrant splice event which occurred within exon 7 of the PDGF-B gene and mimicked the v-*sis* oncogene. Thus, deletion of this region must confer a growth advantage to cells transformed by these retroviruses or be necessary for efficient retrovirus replication.

We investigated the regulation of PDGF-B mRNA expression during megakaryocytic differentiation of K562 cells. Induction by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) led to a greater than 200-fold increase in PDGF-B transcript levels in these cells. Induction was dependent on protein synthesis and was not enhanced by cycloheximide exposure. In our initial investigation of the PDGF-B promoter, a minimal promoter region, which included sequences extending only 42 base pairs upstream of the TATA signal, was found to be as efficient as 4 kilobase pairs upstream of the TATA signal in driving expression of a reporter gene in uninduced K562 cells. We also functionally identified different regulatory sequence elements of the PDGF-B promoter in TPA-induced K562 cells. One region acted as a transcriptional silencer, while another region was shown to bind nuclear factors and was the target for *trans*-activation in normal and tumor cells. In one tumor cell line, which expressed high PDGF-B mRNA levels, the presence of the positive regulatory region resulted in a 30-fold increase in promoter activity. However, the ability of the minimal PDGF-B promoter to drive reporter gene expression in uninduced K562 cells and normal fibroblasts, which contained no detectable PDGF-B transcripts, implies the existence of other negative control mechanisms beyond the regulation of promoter activity.

The transforming activities of PDGF-A and PDGF-B genes were compared. The PDGF-A chain gene was markedly less efficient in inducing transformation than the PDGF-B gene under the influence of the same promoter. There are significant differences in the secretory and growth stimulating properties of the two chains. These properties appear to account for the much more potent transforming ability of the PDGF-B gene. These findings provide insights into biologic properties of a growth factor responsible for potent autocrine stimulation of abnormal cell proliferation.

By use of an appropriately engineered baculovirus expression vector, the v-*sis* protein was expressed in the insect cell line *Spodoptera fungiperida* (Sf9) at a level 50 to 100 fold greater than that observed with overexpression in mammalian cell transfectants. The *sis* protein produced by Sf9 cells underwent processing similar to that observed in mammalian cells including efficient disulfide-linked dimer formation. Moreover, the recombinant *sis* protein was capable of binding PDGF receptors and inducing DNA synthesis as efficiently as PDGF-B synthesized by mammalian cells. A significant fraction of *sis* protein was released from Sf9 cells, which made possible a one-step immunoaffinity purification to near homogeneity with a 40% recovery of biological activity.

These results demonstrate that a protein whose normal processing requires both interchain disulfide bridge formation can be efficiently expressed in a biologically active form in insect cells using a baculovirus vector system.

bFGF. Basic fibroblast growth factor (bFGF) is a potent mitogen for a wide variety of cell types. Unlike most growth factors, the primary translation product for bFGF appears to lack a secretory signal peptide. To explore the normal mode of bFGF release, as well as to investigate the growth factor's oncogenic potential, expression vectors were created for a bFGF cDNA and for a chimeric molecule in which the bFGF coding sequence was linked to the human growth hormone signal peptide sequence. Transfection of NIH/3T3 cells with the bFGF cDNA vectors caused the synthesis of high levels of biologically active, cell-associated bFGF, but no evidence of transformation was detected. In contrast, the chimeric bFGF signal peptide expression vector induced foci of transformation at a very high frequency. The transformed cells grew in soft agar and were tumorigenic in nude mice. The majority of the immunoreactive bFGF species made by the transformed cells was found in the conditioned medium and appeared to be posttranslationally modified, indicating that the chimeric bFGF signal peptide molecule was processed through the secretory pathway. The secreted bFGF exhibited little mitogenic activity, suggesting that interaction of bFGF with its receptor likely occurs while the fusion protein is being processed along the secretory pathway.

TGFα. To investigate the role of transforming growth factor α (TGFα) in tumor development, we introduced the human TGFα (hTGFα) cDNA into cultured primary mouse epidermal cells or papilloma cells using a replication-defective retroviral vector and analyzed skin grafts constructed with such cells. Expression of the exogenous gene was confirmed by detection of hTGFα mRNA by northern RNA blot analysis, and the secreted hTGFα was measured by ELISA of culture supernatants. Tumor cells expressing hTGFα produced benign tumors (papillomas) which were 1.5- to 2-fold larger than tumors of parental cells when tested as skin grafts on nude mice. Grafts of normal cells that expressed hTGFα produced normal skin. When mixtures of parental tumor cells and normal mouse keratinocytes were grafted to nude mice, papilloma formation was suppressed and tumors that did form were small. Grafts of hTGFα-producing papilloma cells combined with either normal epidermal cells or hTGFα-producing epidermal cells yielded large tumors. Mixed grafts containing keratinocytes expressing hTGFα and parental papilloma cells also produced large tumors. While the tumor size was substantially increased by hTGFα expression, the tumors that developed in all groups were histologically benign and reached a stable size 4-5 weeks after grafting. These results indicate that expression of hTGFα by either tumor cells (autocrine) or adjoining normal cells (paracrine) can stimulate tumor growth, particularly when tumor growth is suppressed by normal tissue. However, expression of this growth factor did not appear to influence tumor progression directly.

KGF. A novel growth factor specific for epithelial cells was identified in conditioned medium of a human embryonic lung fibroblast cell line. The factor, provisionally termed keratinocyte growth factor (KGF) because of its predominant activity on this cell type, was purified to homogeneity by a combination of ultrafiltration, heparin-Sepharose affinity chromatography, and hydrophobic chromatography on a C_4 reversed-phase HPLC column. KGF was both acid and heat labile and consisted of a single polypeptide chain of ~28 kiladaltons. Purified KGF was a potent mitogen for epithelial cells, capable of stimulating DNA synthesis in quiescent BALB/MK epidermal keratinocytes by

>500-fold with activity detectable at 0.1 nM and maximal at 1.0 nM. Lack of mitogenic activity on either fibroblasts or endothelial cells indicated that KGF possessed a target cell specificity distinct from any previously characterized growth factor. Microsequencing revealed an amino-terminal sequence containing no significant homology to any known protein. The release of this growth factor by human embryonic fibroblasts raises the possibility that KGF may play a role in mesenchymal stimulation of normal epithelial cell proliferation.

II. Growth Factor Receptors

A genomic sequence and cloned complementary DNA has been identified for a novel receptor-like gene of the PDGF receptor/colony stimulating factor type 1 (PDGF receptor/CSF1) receptor subfamily. The gene recognized a 6.4-kilobase transcript that was coexpressed in normal human tissues with the 5.3-kilobase PDGF receptor mRNA. Introduction of complementary DNA of the novel gene into COS-1 cells led to expression of proteins that were specifically detected with antiserum directed against a predicted peptide. When the new gene was transfected into COS-1 cells, a characteristic pattern of binding of the PDGF isoforms was observed which was different from the pattern observed with the known PDGF receptor. Tyrosine phosphorylation of the receptor in response to the PDGF isoforms was also different from the known receptor. The new PDGF receptor gene was localized to chromosome 4q11-4q12. The existence of genes encoding two PDGF receptors that interact in a distinct manner with three different PDGF isoforms likely confers considerable regulatory flexibility in the functional responses to PDGF.

*erb*B-2. Because of our interest in genes coding for growth factor receptors, we used the v-*erb*B gene to probe for related genes that might be candidates for receptor coding sequences. This led to the discovery of a novel epidermal growth factor (EGF) receptor-related gene, designated *erb*B-2. This gene was found amplified and/or overexpressed in a significant fraction of human mammary adenocarcinomas, and its direct oncogenic potential in vitro was demonstrated.

Our in vitro observations were paralleled by in vivo findings that a number of human mammary tumors and tumor cell lines display overexpression of the *erb*B-2 gene. In addition, we demonstrated that the levels at which the *erb*B-2 protein is capable of inducing an in vitro transformation are comparable to those detected in naturally occurring tumors exhibiting *erb*B-2 overexpression. Recently, an inverse correlation of *erb*B-2 gene amplification and disease-free survival has been reported in breast cancer patients, suggesting a predictive role of *erb*B-2 gene amplification in an aggressive disease course in human mammary neoplasia. Taken together, these observations establish a mechanistic basis for growth factor receptor gene amplification and overexpression as representing a causal driving force in the clonal evolution of a tumor cell rather than being an incidental consequence of tumorigenesis.

Compared with normal *erb*B-2 gp185, mutant *erb*B-2 proteins generated by mutations either in the transmembrane domain or by NH2-terminal deletion are able to transform NIH/3T3 cells at a 10- to 100-fold greater efficiency. Mutant proteins of both classes show increased tyrosine kinase activity, suggesting that an abnormal level of receptor-associated tyrosine kinase activity is a major determinant of *erb*B-2 oncogenic potential.

Epidermal Growth Factor Receptor (EGFR). Alterations affecting the epidermal growth factor (EGF) transforming growth factor α (TGFα)-responsive mitogenic pathway are frequently detected in malignancies. In particular, the EGF receptor (EGFR) molecule has been found overexpressed in a number of human tumors, and TGFα is produced by a large array of tumor cells. Gene transfer experiments have previously demonstrated that expression of either TGFα or EGFR alone is not sufficient to induce the transformed phenotype in NIH/3T3 cells. We sought to investigate the biological effect of expression of TGFα and high levels of EGFR in this model system. We demonstrated that the gene for TGFα acts as a potent oncogene in NIH/3T3 cells overexpressing EGFR (NIH-EGFR, $>10^6$ EGFR). We further show that TGFα directly stimulates proliferation of the cell in which it is produced and provides evidence that the extracellular compartment of the transformed cell is the major site of interaction between TGFα and EGFR. Analysis of human tumor cell lines revealed a strong correlation between expression of TGFα and overexpression of EGFR. Moreover, high levels of EGF-independent tyrosine phosphorylation of the EGFR were detected both in NIH-EGFR expressing TGFα and in high EGFR and TGFα coexpressing human tumor cell lines. Thus, the two events instituting the EGFR/TGFα autocrine loop responsible for transformation in vitro may play a role in the development of some human malignancies.

III. Other Oncogenes

dbl. We isolated cDNA clones representing the human *dbl* proto-oncogene transcript. Nucleotide sequence analysis revealed an open reading frame encoding a predicted protein of 925 amino acids. Using peptide antisera directed against specific proto-*dbl* peptides, a 115-kd protein was detected in COS cells transfected with an expression vector containing the entire coding region of proto-*dbl*. This molecular weight is consistent with that predicted from the open reading frame. We have previously shown that the *dbl* oncogene was generated by substitution of the 5' portion of proto-*dbl* with an unrelated human sequence. In this study we show that this rearrangement resulted in the loss of the 497 amino-terminal codons of the *dbl* proto-oncogene. Under the influence of a strong promoter proto-*dbl* could readily transform NIH/3T3 cells but its transforming activity was less than that of the *dbl* oncogene driven by the same promoter. Proto-*dbl* expression is, therefore, sufficient to transform NIH/3T3 cells, but specific structural alterations of its coding region significantly enhance its transforming activity. No apparent similarity was detected between the predicted proto-*dbl* product and other known proto-oncogenes. However, a stretch of 300 amino acids within the N-terminal half of proto-*dbl* showed structural similarity to the intermediate filament vimentin. This region in proto-*dbl* contains a heptad repeat motif characteristic of an α-helical coiled-coil structure. Taken together, these findings indicate that the human proto-*dbl* represents a new class of cellular oncogenes that may be related to cytoskeletal elements of the cell.

fyn. The *src* gene is the prototype for a family of closely related genes whose products have protein-tyrosine kinase activity. We recently described another member of this family, designated *fyn*, whose cDNA was isolated from normal human fibroblasts. To examine the possible role of *fyn* as an oncogene, we investigated the effects of *fyn* overexpression on NIH/3T3 cells. Our findings demonstrate that normal *fyn* overexpression induces morphologic transformation and anchorage-independent growth. In addition, at relatively low frequency, *fyn* acquired properties of a dominant-acting oncogene capable

of inducing the fully tumorigenic phenotype. Genetic changes associated with the conversion of normal *fyn* cDNA into a transforming gene with high focus-forming activity were localized to the carboxyl-terminal region of its translational product.

IV. Epithelial Cell Models for Normal Growth, Differentiation and Malignancy

BALB/MK epidermal keratinocytes require EGF for growth in serum-containing medium and terminally differentiate in response to high Ca^{++} concentration. Several oncogenic retroviruses have been shown to relieve the EGF requirements and to block calcium-induced terminal differentiation. We developed a chemically defined medium to investigate the minimum growth factor requirements for BALB/MK cells, as well as how such requirements might be altered by retroviral oncogenes. In this medium, insulin, apparently acting as IGF-1 and EGF, supported cell growth in a manner comparable to serum and EGF. Acidic as well as basic fibroblast growth factors (FGF) substituted for EGF but not insulin in supporting BALB/MK proliferation. Infection with retroviruses containing v-*ras* oncogenes (v-H-*ras*, v-K-*ras*), oncogenes derived from growth factor receptors (v-*erb*B, v-*fms*) or the v-*mos* oncogene permitted growth in defined medium containing insulin but lacking EGF. The v-*fgr* oncogene, a member of the *src* subfamily, was unique in conferring independence insulin and EGF. Our findings establish the applicability of this system for biologic assay of epithelial cell growth factors as well as identification of specific growth factor requirements that can be altered or complemented by the actions of specific oncogenes.

Using a defined medium culture system, we investigated the role of physiological concentrations of EGF on phosphoinositide metabolism in these cells. The results show that EGF rapidly activates phospholipase-C-mediated phosphoinositide metabolism resulting in the generation of the second messengers, inositol 1,4,5-trisphosphate and diacylglycerol. These metabolites control intracellular Ca^{++} levels and activate protein kinase C, respectively. Protein kinase C activation in response to EGF was evidenced by the phosphorylation of the acidic 80 kilodalton endogenous protein substrate (p80) specific for this kinase. In contrast, insulin, which acts in concert with EGF to cause BALB/MK cell proliferation, had no effect on phosphoinositide metabolism nor led to any additional stimulation when added in combination with EGF. Taken together, our results show that rapid alterations in phosphoinositide metabolism and protein kinase C activation are associated with the normal mitogenic response of keratinocytes to EGF.

While it is known that BALB/MK keratinocytes require EGF for proliferation and terminally differentiate in response to elevated extracellular Ca^{++} concentrations, the molecular pathways controlling cell differentiation in this system have yet to be established. We showed that a dramatic and sustained activation of phosphoinositide metabolism is produced upon addition of Ca^{++} to BALB/MK cultures. The pattern of inositol trisphosphate isomers released in response to Ca^{++} challenge appeared to be atypical. Inositol 1,3,4-trisphosphate release was observed by 30 seconds and was produced earlier than any alteration in inositol 1,4,5-trisphosphate levels. Concomitant with the liberation of inositol phosphates, an increased production of diacylglycerol was observed. Despite a threefold increase in diacylglycerol levels detected even at 12 hours after Ca^{++} addition, no evidence of functional activation or downregulation of protein kinase C was found. This was established by measuring p80 phosphorylation, EGF binding and protein kinase C levels by immunoblotting. Analysis of the diacylglycerol

generated following Ca^{++} addition to BALB/MK cells revealed that a significant proportion of that lipid is an alkyl-ether-glyceride molecular species. Therefore, it is possible that this diacylglycerol molecular species may play a role in the Ca^{++}-induced differentiation program of BALB/MK cells through mechanisms other than stimulation of classical protein kinase C.

V. Lentiviruses

A full length molecular clone of the equine infectious anemia virus (EIAV) has been obtained. This virus, although unrelated to the DNA of uninfected horses and other species tested, is genetically related to both the retroviruses of acquired immune immunodeficiency syndrome (AIDS) and ovine and caprine lentiviruses in the *gag* and polymerase genes. These observations suggest that these viruses have evolved from a common progenitor and are evolutionarily closely related. The nucleotide sequence of the integrated form of the genome of EIAV has been determined. The molecular analysis of EIAV with respect to the requirements needed to develop a vaccine will not only contribute to control of an important pathogen of horses, but also will represent an important model for the development of vaccines to human retroviruses.

Publications:

Aaronson SA, Falco JP, Taylor WG, Cech AC, Marchese C, Finch PW, Rubin J, Weissman BE, Di Fiore PP. Pathways in which growth factors and oncogenes interact in epithelial cell mitogenic signal transduction. Ann NY Acad Sci (In Press)

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Patents:

Beckmann MP, Betsholtz C, Heldin C-H, Westermark B, Di Fiore PP, Pennington CY, Robbins KC, Aaronson SA. US Patent (Pending): Human PDGF-A and PDGF-B Chains Differ in Their Biological Properties and Transforming Potential.

Eva A, Vecchio G, Rao CD, Tronick S, Aaronson SA. US Patent (Pending): Sequence Analysis of *dbl* cDNA Predicts a Novel Oncogene Product.

King CR, Kraus MH, Aaronson SA. US Patent (Pending): A Human Gene Related to but Distinct from EGF Receptor Gene.

Kruh GD, Aaronson SA. US Patent (Pending): Definition of a Human Gene Related to but Distinct from the Abelson Proto-oncogene.

Lacal JC, Aaronson SA. US Patent (Pending): Deletion Mutants and Monoclonal Antibodies Against *ras* Proteins.

Matsui T, Aaronson SA, Pierce JH. US Patent (Pending): Type α Platelet-Derived Growth Factor receptor Gene.

Miki T, Aaronson SA. US Patent (Pending): Efficient Directional Cloning System to Construct cDNA Libraries Containing Full-length Inserts at a High Frequency.

Rubin JS, Finch PW, Aaronson SA. US Patent (Pending): DNA Encoding a Growth Factor Specific for Epithelial Cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04941-17

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TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemical Characterization of Retroviruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Laboratory of Cellular and Molecular Biology

SECTION

Gene Structure Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

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☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies on animal lentiviruses have led to the isolation of an infectious molecular clone of equine infectious anemia virus (EIAV). The nucleotide sequence of this clone is being determined and the in vivo effects of virus derived from the clone have been studied. The availability of an infectious molecular clone has made possible analysis of the replicative pathway of viral nucleic acids and elucidation of mechanisms by which viral gene expression is controlled. The pattern of viral gene expression in cells infected with EIAV is being studied by using molecular hybridization and cDNA cloning techniques and has led to the identification of transcripts encoding putative viral regulatory genes as well mRNAs that are differentially expressed in acutely and chronically infected cells. Cis-acting elements within the long terminal repeats (LTRs) of EIAV and another lentivirus, caprine arthritis and encephalitis virus (CAEV), have been localized and efforts are underway to identify cellular and viral proteins that may bind to these sequences. A retrovirus (LPDV) that causes lymphoproliferative disease in turkeys was characterized. Although LPDV causes disease rapidly, no evidence for a viral oncogene was obtained. Studies on the evolutionary relationship of LPDV to other retroviruses were also conducted.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
M. Pech	Visiting Scientist	LCMB	NCI
M. Kelley	Medical Staff Fellow	LCMB	NCI
T. Miki	Visiting Scientist	LCMB	NCI

Objectives:

The purposes of this project are to biochemically characterize animal lentiviruses in order to understand the mechanisms by which these viruses cause disease and to develop strategies to prevent and treat lentivirus infection.

Methods Employed:

Standard biochemical techniques for nucleic acid isolation and analysis; assays for enzymes of nucleic acid synthesis and degradation; molecular hybridization techniques to detect and characterize viral genomes; recombinant DNA techniques for the cloning and amplification of retroviral genes; analysis of cloned DNAs by restriction endonuclease mapping, nucleotide sequencing and polymerase chain reaction (PCR); immunological techniques (RIA, Western, and ELISA).

Major Findings:

We have continued the characterization of an infectious molecular clone of the integrated form of the EIAV genome. Virus obtained by transfection of either equine dermis or canine thymus cells with DNA of this clone (designated EIAV clone 22) was inoculated into ponies in collaboration with F. Fuller and L. Coggins at North Carolina State University. No symptoms characteristic of EIA were detected, although the animals mounted a transient, weak immune response to EIAV *gag* antigens. We were unable to detect viral genomes in peripheral blood lymphocytes of the infected animals by using the PCR technique. Laboratory strains of EIAV that have been adapted to grow in culture are known to lose virulence, but pathogenicity can be regained by serial passage of blood from the initial infected horse into an uninfected, healthy animal. When blood from the animal infected with the molecularly cloned virus was passaged in this way, pathogenic effects were not noted. Studies are now in progress to attempt to clone sequences from viruses directly isolated from animals during the peaks of EIA episodes. These genomic segments will be introduced into clone 22 in order to attempt to define sequences that determine pathogenicity. One possible explanation for the failure of clone 22 to induce disease in animals is that its target cell specificity is altered. Using monocyte-macrophage specific growth factors provided by J. Pierce of LCMB, we are investigating the ability of the field strain of EIAV, the standard cell culture-adapted strain of EIAV and virus derived from clone 22 to infect cultures of equine peripheral blood lymphocytes and skin fibroblasts. A molecular clone of integrated EIAV DNA (designated clone 1369) that we had previously characterized was not infectious for either equine dermis or canine thymus in DNA transfection experiments. The only obvious defect in this clone evident by nucleotide sequence analysis was a stop codon

within the *env* open reading frame. In collaboration with F. Fuller, this mutation was repaired; however, it was unable to direct the synthesis of infectious virus particles following DNA transfection. In order to localize the second defect, recombinants between clones 22 and 1369 are being constructed. Once the defect is localized, comparative nucleotide sequence analysis will be performed.

Transcriptional regulatory elements within the EIAV LTR were studied by analysis of deletion mutants. The promoter region of the EIAV LTR was localized to the 3' region of U3 and contained the TATAA promoter concensus sequence. Surprisingly, deletion of regions upstream led to increased transcription. These U3 sequences were found to be required for *trans*-activation of the viral LTR by the EIAV *tat* gene product and could function as a classical enhancer when placed in the context of a heterologous promoter. This region is being mapped in greater detail to exactly define these regulatory elements. The EIAV TAR element was localized to the 5' 40 bases of R. The EIAV TAR element endowed the SV40 early promoter with the ability to be *trans*-activated by the EIAV *tat* gene, and this activity was orientation-dependent. An enhancer element within the U3 region of the CAEV LTR was identified by similar techniques. Despite numerous attempts, we could only demonstrate a threefold *trans*-activation of the CAEV LTR in CAEV-infected cells compared to the 100-fold activation characteristic of other lentiviral LTRs.

In canine cells persistently infected with EIAV viral-specific mRNAs of 8.2 (genomic), 5.4, 4.5 (*env*), 2 and 1.8 kb in size, were detected by hybridization with oligonucleotide probes. The 5.4-kb species consists of *pol*, *env*, and 3' sequences and is present in low abundance. The 1.8 kb species most likely represents the tat gene and the 2 kb species is derived from orf2 and 3' LTR sequences. By cDNA cloning in collaboration with T. Miki, we found that the 2-kb mRNA consists of multiple species. The 1.8 kb transcript was isolated by cDNA cloning as well and its ability to encode a protein that *trans*-activates the viral LTR is being tested. The 5' splice site for all of these transcripts was identified.

The genome of the etiologic agent of lymphoproliferative disease of turkeys was characterized by molecular cloning. The restriction map of the 8-kb genome was determined and its genetic organization was found to be similar to those of replication-competent type C retroviruses. Thus no defective component was identified that might account for the rapid pathogenic effects of this virus. Molecular hybridization experiments uncovered no relatedness between LPDV DNA and known viral oncogenes. Some relatedness to the *pol* gene of RSV and AEV was detected. Since LPDV does not replicate in culture, turkey peripheral blood lymphocytes were transfected with cloned LPDV DNA and then inoculated into turkeys. Lesions typical of LPDV infection were observed and integrated LPDV sequences could be detected in the thymuses. Studies are under way to determine the molecular basis of the rapid pathogenic effects of LPDV.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP04976-12 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Chromatin Radiosensitivity in Cancer Predisposition and Neoplasia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI	K. K. Sanford	Chief, In Vitro Carcinogenesis Sect	LCMB	NCI
Others:	S. Takai	Visiting Fellow	LCMB	NCI
	J. S. Rhim	Microbiologist	LCMB	NCI
	M. Potter	Chief	LG	NCI
	K. H. Kraemer	Research Scientist	LMC	NCI
	R. E. Tarone	Mathematical Statistician	EBP	NCI
	R. Gantt	Chemist	CPCB	NCI
	S. I. Rapoport	Chief	LN	NIA
	T. A. Waldmann	Chief	MET	NCI

COOPERATING UNITS *(if any)*

Howard U. College Med. (R. Parshad); Tel Aviv U. (Y. Shiloh); Walter Reed Dept. of Med. (R. Knight); Univ. of CA, Irvine (E. Stanbridge); Lawrence Berkeley Laboratory (M. Stampfer); Johns Hopkins Hospital (I. Maumenee)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

In Vitro Carcinogenesis Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cultures of skin fibroblasts, peripheral blood lymphocytes and lymphoblastoid cell lines from normal and cancer-prone individuals, as well as neoplastic cells transformed in culture or in vivo, are utilized in evaluating the relationship between radiation-induced chromosomal DNA damage, deficient DNA repair, genetic instability, cancer susceptibility and neoplastic transformation. An increased incidence of chromatid damage after x-irradiation, specifically during G-2 phase of the cell cycle, is associated with both a predisposition to cancer and neoplastic transformation and can provide the basis of a test for genetic susceptibility to cancer. Efforts are currently directed toward developing such an assay using skin fibroblasts, peripheral blood lymphocytes or lymphoblastoid cell lines. A genetic basis for this radiosensitivity with localization of genes to specific chromosomes is indicated from studies with somatic cell hybrids, inbred strains of mice and congenic mouse strains; studies are in progress to localize and map such gene loci. The chromosomal radiosensitivity appears to result from deficient DNA repair during G-2. Another aspect of this project is to develop a reproducible transformation system with human epithelial cells as an in vitro model for following the progression of biologic, cytomorphologic and biochemical changes leading to neoplastic transformation, with particular emphasis on the acquisition of DNA repair deficiencies and genetic instability.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

K. K. Sanford	Chief, In Vitro Carcinogenesis Section	LCMB	NCI
S. Takai	Visiting Fellow	LCMB	NCI
J. S. Rhim	Microbiologist	LCMB	NCI
M. Potter	Chief	LG	NCI
K. H. Kraemer	Scientist	LMC	NCI
R. E. Tarone	Mathematical Statistician	EBP	NCI
R. Gantt	Chemist	CPCB	NCI
S. I. Rapoport	Chief	LN	NIA
T. A. Waldmann	Chief	MET	NCI

Objectives:

The objective of this project is to elucidate, through cell culture studies, mechanisms of neoplastic transformation in human cells. Current emphasis is on the relationship between radiation-induced chromosomal DNA damage, deficient DNA repair, genetic instability, cancer susceptibility and malignant transformation. Efforts are also directed toward developing a transformation system with human epidermal keratinocytes as an in vitro model of human cell carcinogenesis with particular emphasis on the acquisition of DNA repair deficiencies and genetic instability. Specific goals are: (1) to develop an assay for genetic predisposition to cancer using skin fibroblasts, lymphoblastoid cell lines or peripheral blood lymphocytes. This assay will be useful for identifying clinically normal carriers of genes for cancer proneness; (2) to determine at the molecular and cytogenetic levels consequences of radiation-induced chromatin damage and repair during G_2 phase; (3) to determine the relationship between G_2 chromatin repair deficiency, genetic instability and neoplastic transformation in vitro and in vivo; and (4) to identify genes for chromatin repair in mouse and human cells.

Methods Employed:

Chromatid breaks and gaps persisting after G_2 phase x-irradiation (100 R) or exposure for 2 hours to fluorescent light (effective wavelength 405 nm) are quantified in skin fibroblasts, stimulated peripheral blood lymphocytes or lymphoblastoid cell lines. DNA repair inhibitors, caffeine, cytosine arabinoside and b-lapachone are used following irradiation to analyze mechanisms. Since repair of lesions is influenced by the stage of the cell cycle and since the chromatid damage is scored in metaphase cells only, the stage of the cell cycle at the time of irradiation can be experimentally manipulated by varying the interval from treatment to fixation of cells.

Major Findings:

1. Genes on chromosomes 1 and 4 in the mouse are associated with repair of radiation-induced chromatin damage. Early-passage skin fibroblasts from different inbred and congenic strains of mice were x-irradiated (1 Gy), and the number of chromatid breaks was determined at 2.0 hours after irradiation. The cells from DBA/2N, C3H/HeN, STS/A, C57BL/6N, BALB/cJ,

and AKR/N had 25 to 42 chromatid breaks per 100 metaphase cells (efficient repair phenotype). NZB/NJ had >78 and BALB/cAn had 87 to 110 chromatid breaks per 100 cells (inefficient repair phenotype). The BALB/cAn and NZB/NJ mice are the only ones of the eight tested that are susceptible to pristane induction of plasmacytomas. Differences between BALB/cAn and BALB/c.DBA/2 congenic strains which carry less than 1% of the DBA/2 genome indicate that two genes, one on chromosome 4 closely linked to Fv-1, affect the efficiency with which the cells repair radiation-induced chromatin damage. Our data suggest that there may be multiple genes in the mouse that affect the efficiency of DNA repair and susceptibility to pristane-induced plasmacytoma formation. A C.D2 congenic mouse coincidentally has the phenotype of partial resistance to plasmacytoma induction and efficient repair of x-ray induced chromatid breaks. Further analysis will be required to determine if these are controlled by the same or different genes.

2. Skin fibroblasts, peripheral blood lymphocytes or lymphoblastoid cell lines from individuals with genetic disorders predisposing to cancer or with familial cancer show a higher than normal incidence of chromatid breaks and gaps when irradiated during G_2 phase of the cell cycle. The incidence is also higher in human tumor cells and cells transformed in culture than in normal controls. This enhanced G_2 chromatid radiosensitivity is thus associated with both genetic susceptibility to cancer and neoplastic transformation. It is observed only in cells harvested at least 1.5 hours after irradiation. A renewal of culture medium following irradiation is also requisite to disclose this differential response to radiation-induced damage. This abnormal response, associated with genes for cancer-proneness, appears from experimental evidence to result from deficient DNA repair during G_2, a deficiency that accounts for the genetic instability of the cancer cell. In blind studies to date, the response provides a simple direct method for detecting healthy carriers of genes for hereditary dysplastic nevus syndrome, ataxia telangiectasia or xeroderma pigmentosum.

3. Ataxia-telangiectasia (A-T) is an autosomal recessive disease involving chromosomal instability, cancer-proneness and x-ray hypersensitivity. The latter two features are expressed to a limited extent in the heterozygous carriers of the A-T gene(s). Recently we have shown in a blind study that the extent of chromatid damage following G_2 phase x-irradiation of skin fibroblasts in culture is markedly higher in A-T heterozygous cells than in normal controls. This response appears to result from deficient repair of the radiation-induced chromatin damage. We have now applied this test to six additional obligatory heterozygotes and 24 individuals at various risks for being A-T carriers from three Israeli A-T families. In this blind study, with Dr. Shiloh of Israel, six cell lines from the obligatory heterozygotes showed the abnormal response to x-irradiation in G_2, while of the twenty-four cell lines with unknown A-T genotype, 16 showed an A-T heterozygous phenotype and 8responded in a normal way. We concluded that the G_2 response to x-irradiation can be used to assign to these family members presumed A-T heterozygous phenotypes. Furthermore, in these tests to date, the G_2 response behaves as a simple Mendelian dominant trait that segregates with the A-T gene. In one family our test results predicted that a clinically normal child had a 25% probability of developing A-T. We have recently learned that the child is now diagnosed as having A-T.

Publications:

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Sanford KK, Parshad R, Gantt R, Tarone RE, Jones GM. Factors affecting and significance of G_2 chromatin radiosensitivity in predisposition to cancer. Int J Radiat Biol (In Press).

Patents:

Sanford KK, Parshad R, Jones GM. US Patent (Pending): Assay for Genetic Susceptibility to Cancer.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05060-11 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanisms of Carcinogenesis in Vitro: Oncogenic Transformation of Human Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. S. Rhim	Microbiologist	LCMB NCI
Others:	S. A. Aaronson	Chief	LCMB NCI
	P. Arnstein	Veterinary Director	LCMB NCI
	C. Harris	Chief	LHC NCI
	P. Thraves	Volunteer	LCMB NCI
	Z. Salehi	Volunteer	LCMB NCI
	J. Yoo	Guest Researcher	LCMB NCI

COOPERATING UNITS *(if any)*

Erasmus Universitat, Rotterdam, The Netherlands (B. Scholte)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Objectives of this project are (1) to establish and define a cell culture transformation system for identification of carcinogenic agents and humans at high risk for cancer; (2) to develop human cell transformation systems, with particular emphasis on epithelial cells, in order to study host factors regulating cell transformation and the mechanisms of carcinogenesis by chemicals, viruses, hormones and x-irradiation; and (3) to isolate and characterize oncogenes from human tumors.

We have (1) demonstrated the malignant transformation of human primary epithelial cells in culture by the combined action of adenovirus 12 (AD12)-SV40 virus and a variety of retroviral oncogenes; (2) shown the altered response to inducers of terminal squamous differential in human bronchial epithelial cells neoplastically transformed by v-Ki-ras; (3) established salivary gland epithelial cell lines from patients with Sjogren's syndrome and normal individuals in serum-free medium; (4) developed immortalized epithelial cystic fibrosis cell lines by infecting cultured nasal polyp cells with Ad12-SV40 virus; (5) demonstrated the effect of integration and loss of a single v-Ki-ras gene on tumorigenic potential of human osteosarcoma cells; and (6) determined the analysis of p21 as a rapid primary method to screen a large number of tumor materials for the presence of certain types of mutationally activated ras oncogenes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. S. Rhim	Microbiologist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
P. Arnstein	Veterinary Director	LCMB	NCI
C. Harris	Chief	LHC	NCI
P. Thraves	Volunteer	LCMB	NCI
Z. Salehi	Volunteer	LCMB	NCI
J. Yoo	Guest Researcher	LCMB	NCI

Objectives:

1. To develop sensitive in vitro transformation assays to identify carcinogenic agents and humans at high risk for cancer.

2. To develop and study human cell transformation systems, particularly epithelial cells, and the factors regulating cell transformation to elucidate mechanisms of cell transformation by carcinogenic agents and viruses.

3. To search for human oncogenes in human tumors.

Methods Employed:

Biological methods include cell and virus cloning, transformation focus, cell aggregation, soft agar, genome rescue and DNA-mediated gene transfer (DNA transfection) using NIH/3T3 cells as recipients to detect activated cellular transforming genes in malignant tumors. A number of biochemical and molecular biological techniques, including gel electrophoresis, Southern blotting, immunoprecipitation and restriction endonuclease analysis are used to characterize the activated oncogenes. Gene cloning into phage and plasmid is used for characterization of oncogenes.

Major Findings:

Cooperation of v-oncogenes in human epithelial cell transformation. Nontumorigenic human epidermal keratinocytes, immortalized by adenovirus 12 (Ad12)-SV40 virus or pSV3-neo, were transformed by a variety of retroviruses containing *bas*, H-*ras*, *fes*, *fms*, *erb*B and *src* oncogenes. Such transformants showed morphological alterations and induced carcinomas when transplanted into nude mice. These findings demonstrate the malignant transformation of human primary epithelial cells in culture by the combined action of Ad12-SV40 virus and retroviral oncogenes and support a multistep process for neoplastic conversion. This in vitro system may be useful in studying the interaction of a variety of retroviral oncogenes and human epithelial cells.

Human bronchial epithelial cells neoplastically transformed by v-Ki-ras: altered response to inducers of terminal squamous differentiation. Many human bronchial adenocarcinomas have been shown to contain an activated Ki-*ras* oncogene. To test the hypothesis that activated Ki-*ras* may be causally related to human bronchial carcinogenesis, the v-Ki-*ras* oncogene was transferred into an established human bronchial epithelial cell line, BEAS-2B, by infection with Kirsten murine sarcoma virus (Ki-MSV) or by transfection

with a plasmid containing the transforming region of Ki-MSV. These cells formed poorly differentiated adenocarcinomas in athymic nude mice. Cell lines established from these tumors expressed v-Ki-*ras* p21 protein and were highly tumorigenic. Whereas serum or transforming growth factor β_1 induced the BEAS-2B cells at clonal density to undergo growth arrest and squamous differentiation, BEAS-2B cells containing activated *ras* genes were unaffected by transforming growth factor β_1 and were mitogenically stimulated by serum.

Establishment of salivary gland epithelial cell lines from patients with Sjogren's syndrome and normal individuals. Sjogren's syndrome (SS) is an autoimmune disorder characterized by lymphocytic infiltration of salivary and lacrimal glands. An elevated content of Epstein-Barr virus (EBV) in salivary glands of SS patients had been previously demonstrated which suggested that EBV may play a role in pathogenesis. In order to further study the relationship of EBV or the human B-cell leukemia virus (HBLV) to SS, we have attempted to culture epithelial cells from the biopsies of patients with SS in a serum-free medium. We report here successful cultivation of primary epithelial cultures from salivary gland biopsies from patients with SS and normal individuals in a serum-free medium and further establishment of cell lines by introducing the transforming region of SV40 DNA. Characteristics of these cell lines will be studied.

Immortalization of nasal polyp epithelial cells from cystic fibrosis patients. We have developed immortalized epithelial cystic fibrosis (CF) cell lines by infecting cultured nasal polyp cells with an Ad12-SV40 hybrid virus. The cell lines obtained are epithelial in nature as shown by cytokeratin production and morphology, although cytokeratins 4 and 13 typical of primary nasal polyp cells are produced at a much reduced rate. Ussing chamber experiments showed that the precrisis CF cell line NCF3 was able to perform transcellular chloride transport when activated by agents which elevate intracellular calcium. cAMP agonists had no effect on chloride flux in NCF3 as expected for CF cells. The apical chloride channels found with the patch clamp technique in NCF3 and in the postcrisis cell line NCF3A have a conductance similar to that of chloride channels found earlier in normal and CF epithelial cells. The channels show a delay in the onset of activity in off-cell patches and are not activated by increased cAMP levels in the cell. This indicates that immortalized CF epithelial cells will provide a useful model for the study of cystic fibrosis.

Integration and loss of a single v-Ki-*ras* gene affects tumorigenic potential of human osteosarcoma cells. The human osteosarcoma cell line TE85 is not tumorigenic in vivo. Its transformation with Ki-MSV(KHOS) confers full malignant properties and stable nontumorigenic revertants of this KHOS cell line have been obtained. We show that integration and expression of a single copy of the Ki-MSV proviral DNA, which is totally lost in the HOS 240S revertant, is responsible for the acquisition of tumorigenicity. Cytogenetic analysis and the absence of a residual LTR copy in the revertant cellular genome suggest that the loss of Ki-MSV provirus is caused either by chromosomal segregation or by recombination not involving the LTR. In addition, analysis of the expression of *ras* proteins revealed no changes in the pattern of c-*ras* products and the expression of v-*ras* only in the KHOS cells. All these data suggest that TE85 and HOS 240S cell lines could represent a human alternative recipient system to rodent cells in studies with oncogenes.

Detection of *ras* oncogenes by analysis of p21 proteins in human tumor cell lines. To detect mutationally activated *ras* oncogenes, we analyzed electrophoretic mobilities of *ras* p21 proteins utilizing the fact that many *ras* oncogenes produce abnormal p21 proteins that migrate at SDS/polyacrylamide gel electrophoresis as a fast moving or slow moving species in comparison to a normal p21, depending on the kind of mutation. Of 18 human tumor cell lines analyzed, four (SW480, SW620 and SW403 colon cancers, and SW626 ovary cancer) produced p21 belonging to the slow moving spcies, suggesting a point mutation within codon 12 of a member of the three *ras* genes, H-, Ki- and N-*ras*. Subsequent DNA transfection analysis using NIH/3T3 cells as recipients identified activated Ki-*ras* oncogenes in the same four but not in another 14 cell lines. Thus, the analysis of p21 might serve as a rapid primary method to screen a large number of tumor materials for the presence of certain types of mutationally activated *ras* oncogenes.

Publications:

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Reddel RR, Ke Y, Gerwin BI, McMenamin M, Lechner JF, Su R-T, Brash DE, Park J-B, Rhim JS, Harris CC. Transformation of human bronchial epithelial cells by infection with SV40 or adenovirus 12-SV40 hybrid virus, or transfection via stronthium phosphate coprecipitation with a plasmid containing SV40 early region genes. Cancer Res 1988;48:1904-9.

Reddel RR, Ke Y, Kaighn E, Malan-Shibley L, Lechner JF, Rhim JS, Harris CC. Human bronchial epithelial cells neoplastically transformed by v-Ki-ras: altered response to inducers of terminal squamous differentiation. Oncogene Res 1988;3:401-8.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05062-11 LCMB

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transforming Genes of Naturally Occurring and Chemically-Induced Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Eva	Visiting Scientist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
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	D. Ron	Visiting Associate	LCMB	NCI
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LAB/BRANCH
Laboratory of Cellular and Molecular Biology

SECTION
Molecular Genetics Sections

INSTITUTE AND LOCATION
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TOTAL MAN-YEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have isolated cDNA clones of dbl and proto-dbl genes and compared them by transfection assay for their transforming activity on NIH/3T3 cells. We found that proto-dbl overexpression is sufficient to transform NIH/3T3 cells but that specific structural alterations of its coding region significantly enhanced its transforming activity. Comparison of subcellular localization, biogenesis and post-translational modifications of proto-dbl and dbl products indicated that both proteins are cytoplasmic phosphoproteins mainly phosphorylated in serine and present in both cytosol and crude membrane fractions. To understand the biochemical function(s) of the dbl oncogene, we investigated whether dbl-transformed NIH/3T3 fibroblasts showed constitutive alterations of inositol lipid metabolism and found that dbl-transformed cells exhibited increased inositol lipid turnover in response to bradykinin. We have also generated a line of transgenic mice carrying the dbl oncogene and exhibiting a novel phenotype of dominant bilateral lens dysplasia. Finally, we have localized the dbl gene locus on chromosome X just proximal or distal to bands q26-27.2

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. Eva	Visiting Scientist	LCMB	NCI
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W. McBride	Chief, Cellular Regulation Section	LB	NCI
N. Popescu	Research Microbiologist	LB	NCI

Objectives:

Studies are directed to identify transforming genes associated with specific human hematopoietic malignancies. Isolation and characterization of these genes are pursued in order to determine their mechanisms of activation and their specific involvement in the human malignant process.

Methods Employed:

Standard and developmental techniques in cell biology, biochemistry and recombinant DNA are used in these studies.

Major Findings:

Proto-*dbl* and *dbl* oncogene cDNAs were cloned in a eukaryotic expression vector which utilized transcriptional regulatory sequences of the Moloney murine leukemia virus long terminal repeats. Foci were induced by both DNA constructs, but the focus forming activity of the oncogene was found to be 20-50 times higher than its normal counterpart. These data indicated that while overexpression is sufficient to activate proto-*dbl* as a transforming gene, structural alterations affecting the 5' coding sequences can significantly enhance its transforming activity. We thus constructed two deletion mutants, one derived from proto-*dbl* and the second from *dbl* in which only their last 428 amino acids were retained. Under the control of the same promoter, the transforming activity of each of these mutants was similar to that of the *dbl* oncogene. Thus, the loss of the first 497 amino acids of proto-*dbl* is crucial to the high transforming activity of the *dbl* oncogene.

Analysis of *dbl* and proto-*dbl* products revealed that both proteins, p66 and p115, respectively, are primarily associated with the plasma membrane and that these membrane-associated forms are fairly resistant to solubilization by non-ionic detergents, suggesting that both p66 and p115 associate with the cytoskeletal elements of the cell.

We have examined polyphosphoinositide turnover in mouse NIH/3T3 fibroblasts transformed by the *dbl* oncogene as compared to control cells. The *dbl* transformed fibroblasts did not show alterations of the basal level of inositol polyphosphates, polyphosphoinositides, diacylglycerol or phosphatidic

acid. However, *dbl*-transformed NIH/3T3 cells exhibited increased inositol lipid turnover in response to bradykinin and a significantly higher number of bradykinin receptors in *dbl* transfectants as compared to control NIH/3T3 were detected.

We generated a line of transgenic mice carrying the *dbl* oncogene and exhibiting a novel phenotype of dominant bilateral lens dysplasia, which was characterized by an aberrant proliferation and a block of differentiation of the lens epithelial cells. In older eyes the cortex of the lens was replaced by connective tissue and the mice developed microphthalmia. Moreover, the *dbl* protein was found to be specifically expressed in the dysplastic lens, indicating that the expression of the *dbl* gene inhibits the ability of the lens epithelial cells to differentiate into lens fiber cells and may induce transdifferentiation of the epithelial cells into fibroblasts.

The *dbl* oncogene was generated by rearrangements involving three discontinuous regions of the human genome. Analyses of panels of human x rodent somatic cell hybrids demonstrated that the *dbl* gene located on the X chromosome (just proximal or distal to bands q26-27.2) underwent recombination at its 5' and 3' ends with sequences derived from chromosomes 3 (p21-ter) and 16 (p13-q22), respectively. The *dbl* gene was more precisely localized to chromosome Xq27 by *in situ* hybridization. Another oncogene, *mcf*.2, was previously mapped to Xq27 as well. Comparison of the restriction maps of *dbl* and *mcf*.2, taken together with their chromosomal localization, indicates that they may represent the same genetic locus.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05063-11 LCMB

PERIOD COVERED: October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Studies on HHV-6, EBV and HIV

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. V. Ablashi	Microbiologist	LCMB	NCI
Others:	S. Z. Salahuddin	Expert	LTCB	NCI
	R. C. Gallo	Chief	LTCB	NCI
	S. Joseph	Chemist	LTCB	NCI
	F. Wong-Staal	Chief, Mol. Genetics of Hematopoietic Cells Section	LTCB	NCI
	A. Buchbinder	Clinical Associate	LTCB	NCI
	P. Lusso	Visiting Scientist	LTCB	NCI
	P. Levine	Medical Director	FEB	NCI

COOPERATING UNITS (if any)

Harvard U. Med. Sch., Boston, MA (A. Komaroff); U. Cologne, West Germany (G. Krueger); North Shore U. Hosp., Long Island, NY (M. Kaplan); Univ. of Kansas, Kansas City, Kansas (N. Balachandran); Univ. of Nevada (B. Henry)

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Gene Structure Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☑ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided)

The role of human herpesvirus-6 (HHV-6) as a primary etiological agent or as a co-factor in human disease was investigated. Approximately 14% of the heterophile negative infectious mononucleosis (IM) cases are caused by HHV-6. This was evidenced by the detection of IgM antibody to the HHV-6 virus capsid antigen. The contributory role of HHV-6 in chronic fatigue syndrome (CFS) patients was shown when 51% of the patients had elevated antibody to HHV-6 virus capsid antigen (VCA) as compared to age and sex matched healthy donors. Active HHV-6 infection was detected in 9/12 CFS patients' peripheral lymphocyte cells employing HHV-6 monoclonal antibody. Only 1/11 normal donors' lymphocytes contained HHV-6 VCA positive cells. These data were supported by *in situ* hybridization using an HHV-6 DNA probe. Other contributions of HHV-6 have been shown as elevated antibody in Sjogren's syndrome, sarcoidosis, thyroiditis, Hodgkin's disease, B-cell lymphomas and acquired immunodeficiency syndrome (AIDS). Dual infection with HHV-6 and human immunodeficiency virus, type 1 (HIV-1) or HIV-2 of CD-4+ cells (primary or cultured lines) showed enhanced killing, a significant increase in HIV-1 reverse transcriptase activity, and transactivation of HIV LTRs, suggesting that HHV-6 may contribute directly or indirectly to the depletion of CD-4+ T-cells in AIDS. The depletion of helper/inducer cells may lead to more severe immunosuppression, thereby contributing to disease manifestation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. V. Ablashi	Microbiologist	LCMB	NCI
S. Salahuddin	Expert	LTCB	NCI
R. C. Gallo	Chief	LTCB	NCI
S. Joseph	Chemist	LTCB	NCI
F. Wong-Staal	Chief, Mol. Genetics of Hematopoietic Cells Section	LTCB	NCI
A. Buchbinder	Clinical Associate	LTCB	NCI
P. Lusso	Visiting Scientist	LTCB	NCI
P. Levine	Medical Director	EEB	NCI

Objectives:

1. To determine the primary and cofactorial roles of HHV-6 (HBLV) in lymphoproliferative and malignant diseases.

2. To study the interaction of HHV-6, HIV-1 and HIV-2 in order to understand their roles in AIDS and AIDS-related complexes.

Methods Employed:

Primary cell cultures were established from peripheral blood lymphocytes and tumor tissues from AIDS and other patients with tumors and lymphoproliferative disorders and were used for virus isolation and infection with HIV-1, HBLV and Epstein-Barr virus (EBV). Continuous human B- and T-cell lines were also used for propagation of EBV, HIV, and HHV-6. Human cord blood mononuclear cells were used for infecting viral stocks and for use in immunologic assays. Monoclonal and polyclonal antibodies to HIV and human herpesviruses were used in immunologic assays. Biochemical and molecular studies were performed for characterization of virus isolates and detection of viral genomes.

Major Findings:

1. Since the initial isolation of HHV-6 (HBLV) by us in 1986, from lymphoproliferative disease patients and AIDS, a worldwide search has been going on to link this virus to human disease. Yamanishi *et al.*, 1988, reported that *Exanthem Subitum* in infants is caused by HHV-6. We also found that young children suffering from high fever, enlarged lymph glands, muscle pain, headache and a general body rash possessed IgM antibody to HHV-6 VCA, suggesting that young children with a primary HHV-6 infection could suffer a condition similar to that of infants. Moreover, 231 sera from acute infectious mononucleosis (IM) patients revealed that 14.3% (33/231) had elevated IgM antibody to HHV-6 VCA. This suggests that mononucleosis-like illness with atypical lymphocytosis, where EBV and CMV tests are non-diagnostic, are caused by HHV-6. All 33 cases were heterophile antibody-negative and exhibited IM symptoms indistinguishable from heterophile-positive IM cases known to be caused by EBV.

2. The role of HHV-6 in chronic fatigue syndrome (CFS), also known as post-viral fatigue syndrome (PVFS), was investigated. CFS was originally thought to be caused by EBV. This was based on the finding of antibody

to EBV early antigen (EA) and elevated antibody to EBV VCA. However, only a few CFS sera showed this activity. We tested 300 CFS sera and 50 age and sex matched sera from healthy donors for HHV-6 and EBV antibody by indirect immunofluorescence assay (IFA). Eighty (25.8%) of the sera had elevated EBV VCA and EA antibody only; 159 sera (51.3%) had HHV-6 VCA antibody at >1:160 and the majority of these sera had titers ranging from 320-≥10,000. About 20% (61 sera) had elevated antibody titer to both EBV and HHV-6. Healthy donors' sera titers for EBV and HHV-6 were in the normal range (1:20-1:80), suggesting that elevated antibody titer could be the result of virus reactivation. The reactivation of the virus in CFS patient's peripheral blood mononuclear cells was examined after the cells were mitogen stimulated. The stimulated cells were tested for HHV-6 antigen by using monoclonal antibody to HHV-6 and by *in situ* hybridization, using HHV-6 and EBV DNA probes. Of 12 CFS patients' lymphocytes, 9 contained HHV-6 antigen-positive cells which varied from 4-≥35%. These cells were usually single, large and irregular in shape. One of eleven normal donor's lymphocyte samples contained <1-2% HHV-6 antigen-positive cells. The *in situ* data showed that HHV-6 DNA was present in the large cells. Moreover, EBV DNA was also detected in some lymphocyte samples from CFS patients with elevated antibody to EBV and HHV-6. These findings support the fact that in CFS patients not only is the reactivation of the virus evident, but also the number of reactivated cells is significantly high. It is also evident that the interaction of EBV and HHV-6 may contribute to disease manifestation when these viruses are reactivated by other factors.

3. Recent data on prevalence and antibody titer to HHV-6 showed that 60% of the normal donors from the United States, Europe and Canada had HHV-6 antibody. The antibody titer in 95% of the normal donors was ≤1:80. The antibody titer and prevalence rate (87%) was higher in West Africa (≥1:80-1:160). Elevated antibody titer was observed in sarcoidosis (72%), non-EBV and CMV infectious mononucleosis (82%), thyroiditis (83.5%), Hodgkin's disease (80%), Sjogren's syndrome (72%), African Burkitt's lymphoma (88%), other B-cell lymphomas (70%) and acute lymphocytic leukemia (73%). In all these diseases, the antibody titers were elevated (≥1:160-≥1:5120). Antibody titer was not elevated in nasopharyngeal carcinomas or in EBV and CMV infectious mononucleosis, which are known to be associated to EBV. How these elevated antibody titers contribute to the disease manifestation is yet to be investigated.

4. Elevated antibody titers to HHV-6 were observed in 70% of sera from asymptomatic HHV-1 antibody positive patients and AIDS. The titers were highest in AIDS with Kaposi's sarcoma patients. The *in vitro* interaction of HIV-1, HIV-2 and HHV-6 was investigated in order to understand how HHV-6 could contribute to the clinical manifestation of AIDS. Dual infection with HHV-6 and HIV-1 or HIV-2 of fresh CS_4^+ cells obtained from bone marrow, thymus or cord blood mononuclear cells led to enhanced killing and more release of HIV. This was evident by a significant increase in reverse transcriptase activity, transactivation of HIV-1 long terminal repeats and replication of HHV-6 and HIV-1 in the same CS_4^+ cell. This finding was also supported by the dual infection of CF_4^+ T-cell lines. Moreover, using PCR assay, 82% of AIDS peripheral lymphocytes contained HHV-6 DNA. Peripheral lymphocytes from three AIDS

patients exhibited 15-25% HHV-6 late antigen expressing cells, which suggests that a direct interaction occurs between HIV-1 or HIV-2 and HHV-6. This interaction of HIV and HHV-6 *in vivo* could lead to more severe immune suppression, thereby contributing to disease manifestations.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05164-09 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Oncogenes, Growth Factor Pathways and Hematopoietic Cell Signal Transduction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. H. Pierce	Research Microbiologist	LCMB	NCI
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COOPERATING UNITS *(if any)*

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SECTION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We identified and cloned the cDNA of a novel human platelet-derived growth factor receptor (PDGFR), designated the alpha PDGFR, which binds all three PDGF isoforms and undergoes tyrosine autophosphorylation in response to each. By contrast, the previously identified PDGFR, designed the beta receptor, was preferentially tyrosine phosphorylated in response to the PDGF-BB isoform. In order to investigate functional responses mediated by each receptor, we independently expressed their cDNAs in the 32D hematopoietic cell line devoid of either receptor and normally dependent upon interleukin-3 (IL-3) for survival and proliferation. We demonstrated that either receptor transfected into 32D cells could independently mediate major known PDGF activities including mitogenic signal transduction, chemotaxis, and stimulation of phosphoinositide turnover. These results suggest that the major level of regulation of PDGF-induced functional responses must reside in the relative affinities of the three PDGF isoforms for either receptor.

We demonstrated that cross linkage of FcεRI receptors on a series of nontransformed murine mast cell lines or treatment of these cells with calcium ionophores stimulated increased mRNA levels and secretion of a group of lymphokines classically produced by a subset of murine T-cell lines. These factors include IL-3 (a mast cell growth factor), IL-4 (an IgE "switch factor"), IL-5 (an eosinophil differentiation factor) and IL-6 (a factor controlling immunoglobulin secretion. The production of these polypeptide factors by activated mast cells may have great importance in the induction of allergic and antiparasitic inflammatory responses.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. H. Pierce	Microbiologist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
P. P. Di Fiore	Visiting Scientist	LCMB	NCI
M. Ruggiero	Visiting Scientist	LCMB	NCI
O. Segatto	Visiting Associate	LCMB	NCI
M. Kraus	Visiting Scientist	LCMB	NCI
T. Fleming	NRSA Fellow	LCMB	NCI
T. Matsui	Visiting Fellow	LCMB	NCI
E. De Marco	Visiting Fellow	LCMB	NCI
M. Plaut	Guest Researcher	LI	NIAID
W. Paul	Chief	LI	NIAID

Objectives:

(a) To determine mechanisms by which oncogenes abrogate growth factor dependence and induce transformation of factor-dependent hematopoietic cells; (b) to determine the role of growth factors and growth factor receptor expression on the signal transduction pathway controlling either growth or differentiation in cells of connective tissue or hematopoietic origin; and (c) to determine the role of lymphokine activation in the allergic response.

Methods Employed:

Standard hematopoietic culture techniques, including an in vitro hematopoietic colony-forming assay developed to detect transformation of hematopoietic cells by retroviruses, use of feeder layers to establish continuous cell lines, and cloning of established cell lines in soft agar. Other procedures included generation of growth factors and retrovirus infection of factor-dependent cell lines.

Identification of hematopoietic phenotype of retrovirus-transformed hematopoietic cells was performed utilizing histochemical staining, immunofluorescence techniques, radioimmunoassays and enzymatic assays.

DNA transfection by the calcium phosphate and electroporation methods were utilized to identify growth factor receptor-related oncogenes. Analysis of transformants was performed by Southern and northern hybridization analysis, radioimmunoprecipitation and growth factor-binding and mitogenic assays.

Major Findings:

We identified a genomic sequence and cloned cDNA of a novel receptor-like gene of the platelet-derived growth factor receptor (PDGFR)/CSF-1 receptor subfamily. The gene was localized to chromosome 4q11-12 and gave rise to a 6.4-kb transcript that was coexpressed in normal human tissues with the 5.3-kb PDGFR mRNA.

In order to investigate specific functions mediated by the products of two independent PDGFR genes, we used a strategy involving introduction of expression vectors for α and β PDGFR cDNAs into a naive interleukin-3 (IL-3)-dependent hematopoietic cell line, 32D. We demonstrated that each receptor

was able to independently couple with mitogenic signal transduction pathways inherently present in these cells. Moreover, both receptors were capable of inducing a readily detectable chemotactic response. Finally, activation of either receptor led to rapid alterations in inositol lipid metabolism and the mobilization of intracellular Ca^{++}. All of these findings establish that the major biological and biochemical responses observed in cells normally triggered by PDGF can be reconstituted in these hematopoietic cells by expression of either α or β PDGFRs.

PDGF consists of AA, AB and BB isoforms, which arise as dimeric products of two independent PDGF genes. The PDGF-A chain is more ubiquitously expressed by normal cell types than the PDGF-B chain and is more actively secreted as well. Our present results indicate that a major level of regulation of the spectrum of PDGF functional responses resides in the relative affinities of the three PDGF isoforms for either receptor.

Mast cells store a variety of mediators which are secreted upon appropriate stimulation such as cross-linkage of FcεRI. To determine whether lymphokines are also secreted by mast cell stimulation and to verify the biological identification of these lymphokines, we examined the expression of lymphokine mRNA in IGE-sensitized, IL-3-dependent murine mast cells stimulated with antigen or ionomycin. Poly A+ mRNA was prepared from mast cells two hours after stimulation and tested by northern analysis. Resting cells did not express active mRNA for IL-3 or IL-5 and expressed very low levels of IL-4 mRNA and IL-6 mRNA. Cross-linkage of FcεRI on murine mast cells caused a modest increase in IL-4 mRNA and substantial increases in IL-3, IL-5 and IL-6 mRNA. Ionomycin-stimulated cells expressed large amounts of IL-3, IL-4, IL-5 and IL-6 mRNA.

These results indicate that nontransformed long term mast cell lines produce a set of lymphokines, generally considered to be principally derived from T-cells, as a result either of cross-linking of FcεRI or exposure to calcium ionophores. The lymphokines produced by mast cell lines appear well adapted to participate in allergic inflammatory responses. IL-4 has been clearly shown to be critical for IgE production both in vitro and in vivo, while IL-5 is a potent eosinophil differentiation and activation factor. Both IL-3 and IL-4 have roles as stimulants of the growth of mast cells, possibly of both the mucosal and connective tissue types. Thus, production of these lymphokines by activated mast cell lines suggests that production of these factors play an important role in allergic and antiparasitic responses.

Publications:

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Matsui T, Heidaran M, Miki T, Popescu N, LaRochelle W, Kraus M, Pierce J, Aaronson SA. Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. Science 1989;243:800-4.

Mushinski JF, Mountz JD, Pierce JH, Pumphrey JG, Skurla RM Jr, Finkelman FD, Givol D, Davidson WF. Expression of the murine proto-oncogene *bcl-2* is stage specific and cell-type specific. Curr Top Microbiol Immunol 1988;141:333-6.

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Plaut M, Pierce J, Watson C, Hanley-Hyde J, Nordan R, Takak S, Takatsu K, Paul WE. Mouse mast cell lines produce interleukins in response to cross linkage of FcεRI or to calcium ionophores. Nature (In Press)

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Rhim JS, Kawakami T, Pierce J, Sanford K, Arnstein. Cooperation of v-oncogenes in human epithelial cell transformation. Leukemia 1988;2:151S-9S.

Segatto O, King CR, Pierce JH, Di Fiore PP, Aaronson SA. Different structural alterations upregulate in vitro tyrosine kinase activity and transforming potency of the *erb*B-2 gene. Mol Cell Biol 1988;8:5570-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05366-06 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Role of Proto-oncogenes Encoding Growth Factor Receptors in Neoplasia

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Matthias H. Kraus	Visiting Scientist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	L. Marazzi	Visiting Fellow	LCMB	NCI
	A. DiDonato	Visiting Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2 Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

A new member of the erbB/epidermal growth factor receptor (EGF-R) proto-oncogene family was detected by Southern blot hybridization under reduced stringency conditions using v-erbB as a probe. Characterization of the cloned DNA fragment mapped the region of v-erbB homology to three exons with closest homology of 64% and 67% to a contiguous region within the tyrosine kinase domains of the predicted EGF-R and erbB-2 proteins, respectively. Expression of a gene-specific transcript of 6.2 kb was detected in cells of epithelial origin. In order to identify the complete coding structure, cDNA clones were isolated and subjected to nucleotide sequence analysis predicting a transmembrane polypeptide possessing closer structural similarity to both the EGF-R and erbB-2 than to any other known tyrosine kinase. These findings as well as its conserved exon structure implies a common ancestral origin of these three genes, prompting us to designate the new gene as erbB-3. The presence of a putative ligand-binding domain suggests that erbB-3 may represent a novel growth factor receptor molecule for an as yet unidentified ligand. Elevated erbB-3 mRNA levels were demonstrated in certain human epithelial-derived tumor cell lines in comparison with normal cells of the same tissue origin suggesting that increased expression of this novel tyrosine kinase receptor-like molecule, as in the case of EGF-R and erbB-2, may play a role in certain human malignancies. Utilizing human EGF-R, erbB-2 or erbB-3 probes under reduced hybridization stringency, the restriction fragment of a related gene distinct from these genes was detected in normal human genomic DNA. Preliminary molecular characterization of the cloned fragment identified an exon sequence exhibiting closest homology and conserved exon junctions with members of the EGF-R family, suggesting that evolutionary divergence gave rise to yet another member of this tyrosine kinase subfamily.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. H. Kraus	Visiting Scientist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
L. Marazzi	Visiting Fellow	LCMB	NCI
A. Di Donato	Visiting Fellow	LCMB	NCI

Objectives:

1. Identification and characterization of novel proto-oncogenes encoding growth factor receptors based on nucleotide sequence and structural homology to human EGF-R genes.

2. Identification of the normal protein and its functions in normal cell growth and differentiation.

3. Investigation of mechanisms activating such genes in transformed cells and role of activated versions of growth factor receptor-encoding proto-oncogenes in human neoplasia.

Methods Employed:

Southern blotting, northern blotting, dot blot analysis, RNAse protection, recombinant DNA technology including genomic and cDNA cloning, nucleotide sequence analysis, generation of polyclonal peptide antisera and gene product analysis by immunoblotting and immunoprecipitation, detection of *in vivo* phosphorylation, autokinase assays, DNA transfection.

Major Findings:

1. Identification of a novel *erb*B related proto-oncogene, *erb*B-3, in normal genomic DNA.

2. Identification of a gene specific mRNA in epithelial-derived cells.

3. Isolation of complementary DNA encompassing the entire coding sequence of *erb*B-3.

4. Characterization of the *erb*B-3 coding structure predicts a membrane receptor tyrosine kinase of closest homology and common ancestral origin with members of the *erb*B/epidermal growth factor receptor (EGF-R) subfamily of growth factor receptors.

5. Evidence for *erb*B-3 mRNA overexpression in human epithelial derived tumor cells.

6. Identification of an *erb*B-related human gene fragment distinct from EGF-R, *erb*B-2 and *erb*B-3.

Publications:

Di Marco E, Pierce JH, Fleming TP, Kraus MH, Molloy CJ, Aaronson,SA, Di Fiore PP. Autocrine interaction between TGFα and the EGF-receptor: quantitative requirements for induction of the malignant phenotype. Oncogene (In Press)

Fujita J, Kraus MH, Onone H, Srivastava SK, Ebi Y, Kitamura T, Rhim JS. Activated H-*ras* oncogenes in human kidney tumors. Cancer Res 1988;48:5251-55.

King CR, Di Fiore PP, Pierce JH, Segatto O, Kraus MH, Aaronson SA. Oncogenic potential of the *erb*B-2 gene: frequent over-expression in human mammary adenocarcinomas and induction of transformation in vitro. In Lippman ME, ed. Growth regulation of cancer. Proceedings of UCLA symposium. New York: Alan R. Liss, 1988;189-99.

Kraus MH. The oncogenic potential of membrane receptor proteins encoded by members of the human *erb*B proto-oncogene family. In: Ceriani RL, ed. Immunological approaches to the diagnosis and therapy of breast cancer, II (In Press)

Kraus MH, Di Fiore PP, Pierce JH, Aaronson SA. Different mechanisms are responsible for oncogene activation in human mammary neoplasia. In: Lippman M E, Dickson B, eds. Breast cancer: cellular and molecular biology. Boston: Martinus Nijhoff 1988;49-66.

Kraus MH, Di Fiore PP, Pierce JH, Robbins KC, Aaronson SA. Overexpression of proto-oncogenes encoding growth factor or growth factor receptor proteins in malignancy. Cancer Cells, vol VII (In Press)

Kraus MH, Pierce JH, Fleming TP, Robbins KC, DiFiore PP, Aaronson SA. Mechanisms by which genes encoding growth factors and growth factor receptors contribute to malignant transformation. In: Galeotti T, Cittadini A, Neri G, Scarpa A, eds. Membrane in cancer cells. New York: Ann NY Acad Sci 1988;320-36.

Lacroix H, Iglehart D, Skinner M, Kraus MH. Overexpression of *erb*B-2 or EGF receptor proteins present in early stage mammary carcinoma is detected simultaneously in matched primary tumors and regional metastases. Oncogene 1989;4:145-51.

Matsui T, Heidaran M, Miki T, Popescu N, La Rochelle W, Kraus M, Pierce J, Aaronson SA. Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. Science 1989;243:800-4.

Needleman SW, Devine SE, Kraus MH. 12th codon mutation resulting in c-N-*ras* activation in acute myelogenous leukemia. Leukemia 1988;2:91-3.

Popescu NC, King CR, Kraus MH. Localization of the human *erb*B-2 gene on normal and rearranged chromosomes 17 to bands q12-21.32. Genomics 1989;4:362-6.

Patents:

King CR, Kraus MH, Aaronson SA. US Patent (Pending): A Human Gene Related to but Distinct From EGF Receptor Gene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05457-05 LCMB

PERIOD COVERED: October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Growth Factor Receptors in Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. P. Di Fiore	Visiting Scientist	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	J. H. Pierce	Microbiologist	LCMB	NCI
	O. Segatto	Visiting Associate	LCMB	NCI
	F. Lonardo	Visiting Fellow	LCMB	NCI
	E. Di Marco	Visiting Fellow	LCMB	NCI
	F. Fazioli	Guest Researcher	LCMB	NCI

COOPERATING UNITS *(if any)*

None

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Laboratory of Cellular and Molecular Biology

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Molecular Biology Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
5.0	5.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

1. The combined action of transforming growth factor alpha (TGFα) and epidermal growth factor receptor (EGFR) expression on the transformed phenotype of cell lines in culture has been studied. Results indicate that transformation of NIH/3T3 cells by TGFα is dependent on the number of EGFRs expressed. Human tumors overexpressing EGFR also express TGFα and the EGFR shows sign of autocrine activation (high levels of tyrosine phosphorylation in the absence of exogenous ligand stimulator).

2. Chimeric molecules engineered between EGFR and erbB-2 helped dissect the function of two domains of the intracellular portion of these receptors. Our findings indicate that the highly conserved tyrosine kinase (TK) region is responsible for binding of specific intracellular substrates, while the carboxyl terminal (COOH) domain distal to the TK regulates the receptor intrinsic kinase activity and its biological potency.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. P. Di Fiore	Visiting Scientist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. H. Pierce	Microbiologist	LCMB	NCI
O. Segatto	Visiting Fellow	LCMB	NCI
F. Lonardo	Visiting Fellow	LCMB	NCI
E. DiMarco	Visiting Fellow	LCMB	NCI
F. Fazioli	Guest Researcher	LCMB	NCI

Objectives:

1. To study the mechanisms of oncogenic activation of genes which normally encode for growth factor receptors. This will lead to a better understanding of how chronically activated mitogenic signals can convert cells from the normal to malignant state.

2. To study how the above "activated" genes alter the growth properties and the differentiated program of certain cell cytotypes, like hematopoietic and epithelial cells.

3. To study the mechanisms of mitogenic signal transduction and isolate intracellular substrates for the tyrosine kinase activity of growth factor receptors.

Methods Employed:

Generation of eukaryotic expression vectors; gene transfer by DNA transfection (Ca^{++} precipitate method or electroporation); standard recombinant DNA and protein analysis methods; affinity purification of putative growth factor receptor substrates by chromatography on immobilized anti-phosphotyrosine antibodies.

Major Findings:

Genetic alterations affecting the epidermal growth factor (EGF)/transforming growth factor α (TGFα)-responsive mitogenic pathway are frequently detected in experimentally induced and naturally occurring malignancies. In particular, the EGF-receptor (EGFR) molecule has been found overexpressed in a number of human tumors, and TGFα is produced by a large array of tumor cells. Gene transfer experiments have previously demonstrated that expression of either TGFα or EGFR alone is not sufficient to induce the transformed phenotype in NIH/3T3 cells. In this study we sought to investigate the biological effect of expression of TGFα and high levels of EGFR in this model system. We demonstrate that the gene for TGFα acts as a potent oncogene in NIH/3T3 cells overexpressing EGFR (NIH-EGFR, $>10^6$ EGFR). We further show that TGFα directly stimulates proliferation of the cell in which it is produced and provide evidence that the extracellular compartment of the transformed cell is the major site of interaction between TGFα and EGFR. Analysis of a series of human tumor cell lines revealed a strong correlation between expression of TGFα and overexpression of EGFR. Moreover, high levels of EGF-independent tyrosine phosphorylation of the EGFR were detected both in NIH-EGFR expressing TGFα (NIH-EGFR-TGFα) and in high EGFR and TGFα coexpressing

human tumor cell lines. Thus, the two events instituting the EGFR-TGFα autocrine loop responsible for transformation in vitro may play a role in the development of some human malignancies.

The epidermal growth factor (EGF) receptor and *erb*B-2 genes encode structurally related proteins whose overexpression has been implicated in the pathogenesis of certain human malignancies. Previous studies have shown that the EGF receptor (EGFR) can efficiently couple with mitogenic signalling pathways in 32D hematopoietic cells normally devoid of EGF, arguing that a major control of growth regulation is at the level of receptor expression and ligand availability. When expression vectors for *erb*B-2 or its activated truncated counterpart, Δ*Nerb*B-2, were introduced into 32D cells, neither was capable of inducing proliferation despite overexpression and constitutive enzymatic activity of their products at levels associated with potent transformation of fibroblast target cells. Thus, the EGFR and *erb*B-2 couple with distinct mitogenic signalling pathways. By means of chimeric molecules engineered between the EGFR and *erb*B-2 cDNAs, we localized the region responsible for the observed specificity of intracellular signal transduction to a 300-amino acid stretch encompassing their conserved tyrosine kinase and transmembrane domains. In addition we demonstrated, by the same chimeric molecules approach, that the carboxy-terminal domain of *erb*B-2 and of its cognate molecule, the EGFR, participates in the regulation of intrinsic receptor tyrosine kinase and biological activities.

Publications:

Aaronson SA, Falco JP, Taylor WG, Cech AC, Marchese C, Finch PW, Rubin J, Weissman BE, Di Fiore PP. Pathways in which growth factors and oncogenes interact in epithelial cell mitogenic signal transduction. In: Galeotti T, Cittidin A, Mori G, Scarpa A, eds. Membrane in cancer cells. New York: Ann NY Acad Sci 1988;320-36.

Beckmann MP, Betsholtz C, Heldin C-H, Westermark B, Di Fiore PP, Robbins KC, Aaronson SA. Human PDGF-A and PDGF-B chain genes differ in their biological properties and transforming potential. Science 1988;241:1346-9.

Di Marco E, Pierce JH, Fleming TP, Kraus MH, Molloy CJ, Aaronson SA, Di Fiore PP. Autocrine interaction between TGFa and the EGF-receptor: quantitative requirements for induction of the malignant phenotype. Oncogene (In Press).

Falco JP, Taylor WG, Di Fiore PP, Weissman BE, Aaronson SA. Interactions of growth factors and retroviral oncogenes with mitogenic signal transduction pathways of BALB/MK keratinocytes. Oncogene 1988;2:573-8.

King CR, Di Fiore PP, Pierce JH, Segatto O, Kraus MH, Aaronson SA. Oncogenic potential of the *erb*B-2 gene: frequent overexpression in human mammary adenocarcinomas and induction of transformation *in vitro*. In: Lippman ME, ed. Growth regulation of cancer. Proceedings of UCLA symposium. New York: Alan R. Liss, Inc 1988;189-99.

Kraus MH, Di Fiore PP, Pierce JH, Aaronson SA. Different mechanisms are responsible for oncogene activation in human mammary neoplasia. In: Lippman ME, Dickson B, eds. Breast cancer: cellular and molecular biology. Boston: Martinus-Nijhoff 1988;49-66.

Kraus MH, Di Fiore PP, Pierce JH, Robbins KC, Aaronson SA. Overexpression of proto-oncogenes encoding growth factor or growth factor receptor proteins in malignancy. Cancer Cells, vol 7, New York: Cold Spring Harbor (In Press).

Kraus MH, Pierce JH, Fleming TP, Robbins KC, Di Fiore PP, Aaronson SA. Mechanisms by which genes encoding growth factors and growth factor receptors contribute to malignant transformation. In: Galeotti T, Cittadini A, Neri G, Scarpa A, eds. Membrane in cancer cells. New York: Ann NY Acad Sci 1988;320-36.

Segatto O, King CR, Pierce JH, Di Fiore PP, Aaronson SA. Different structural alterations upregulate *in vitro* tyrosine kinase activity and transforming potency of the *erb*B-2 gene. Mol Cell Biol 1988;8:5570-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05461-05 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Mechanism of Activation of dbl Oncogene: Structural/Functional Analysis of KGF

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. Ron	Visiting Associate	LCMB	NCI
Others:	A. Eva	Visiting Scientist	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The activation of the dbl oncogene involved the loss of the first 497 amino acids of its normal counterpart and the acquisition of a new N-terminus of 50 amino acids. By generating N-terminal truncated mutants, we showed that the loss of the 497 amino acids from proto-dbl rather than the acquisition of a new N-terminus is crucial for dbl activation. This data suggested that sequences within the N-terminal region of proto-dbl down regulate proto-dbl transforming potential. To identify these sequences, we constructed deletion mutants lacking different regions within proto-dbl's first 497 amino acids. We found that the sequences responsible for this down regulation are localized within amino acids 432-497. This same region seems to affect proto-dbl turnover since its removal increases the half life of the mutant protein. Recently, we have found that a region of 280 amino acids within the region of proto-dbl which is required for its transforming activity has a statistically significant similarity to the yeast Saccharomyces cerevisiae cell cycle protein CDC24. We therefore examined the effect of small deletions within this region on the focus forming activity of proto-dbl, as well as deletions outside of this region starting from the C-terminus of proto-dbl. The results indicated that every deletion within the region of similarity with CDC24 abolished proto-dbl transforming activity, while removal of 100 amino acids from its C-terminus had no effect on this activity. Studies were also initiated on KGF, a new keratinocyte specific growth factor discovered and characterized in our laboratory. These studies are aimed at the understanding of KGF's normal role by examining its pattern of expression in an animal model system at different stages of development. In addition, the domains required for KGF interaction with its target cells are being characterized.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. Ron	Visiting Associate	LCMB	NCI
A. Eva	Visiting Scientist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI

Objectives:

1. To further narrow down the minimum region required for down regulation of proto-*dbl* transforming activity using in vitro mutagenesis techniques.

2. To assess the role of membrane binding of the proto-*dbl* product on its transforming function.

3. To extend the studies of KGF pattern of expression in an animal model system and to utilize *in situ* hybridization to examine the specific site of KGF expression.

4. To study the role of KGF in tumorigenesis by assessing its level of expression in human tumors.

5. To express KGF in prokaryotic and eukaryotic expression vectors. This is particularly important since a high level of KGF is crucial to study its interaction with its putative receptor and its affect on cells from different lineages and possibly in vivo.

6. To generate KGF mutants utilizing in vitro mutagenesis for studies involving identification of KGF domains required for its target cell specificity.

Methods Employed:

In vitro mutagenesis to construct proto-*dbl* deletion mutants; standard methods of RNA extraction from tissue and cell lines; northern blotting. cDNA library screening, as well as other molecular cloning techniques such as dideoxy sequencing technique, Southern blotting, restriction enzyme analysis, construction of eukaryotic and prokaryotic expression vectors, transfection, gel electrophoresis, preparation of high molecular weight DNA. Radioimmuno-precipitation and western blotting.

Major Findings:

1. The N-terminal region of proto-*dbl* down regulates its transforming activity. The sequence responsible for this down regulation is localized within 60 amino acids just upstream to the *dbl* oncogene breakpoint.

2. Increased transforming activity of *dbl* is associated with higher stability of its product. The same 60 amino acids required to down regulate the transforming activity of *dbl* seems to determine proto-*dbl* stability.

3. The minimum transforming region of proto-*dbl* is localized within amino acids 497-825.

4. Any deletion within the region of similarity between proto-*dbl* and CDC24 completely abolishes *dbl* focus forming activity.

5. KGF is expressed in the skin and lungs of newborn mice. This finding is interesting since KGF was initially discovered and isolated from human embryo fibroblasts and found in vitro to specifically enhance the growth of keratinocytes.

Publications:

Eva A, Srivastava S, Vecchio G, Ron D, Tronick S, Aaronson SA. Biochemical characterization of *dbl* oncogene and its product. In: Tonini GP, Massimo L, Cornaglia-Ferraris P, eds. Oncogenes in pediatric tumors. London/New York: Harwood Academic, 1988;4:215-31.

Ron D, Tronick SR, Aaronson SA, Eva A. Molecular cloning and characterization of the human *dbl* proto-oncogene: evidence that its overexpression is sufficient to transform NIH/3T3 cells. EMBO J 1988;7:2465-73.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05463-05 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Participation of Growth Factors and Oncogene Products in Growth Regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	W. G. Taylor	Biologist	LCMB NCI
Others:	S. A. Aaronson	Chief	LCMB NCI
	J. S. Rubin	Biotechnology Fellow	LCMB NCI

COOPERATING UNITS *(if any)*

Johns Hopkins Oncology Center, Baltimore, MD (J. Falco); Childrens Hospital of Los Angeles, CA (B. E. Weissman)

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The long-term objective of this program is to understand the mechanism(s) of cellular changes fundamental to neoplastic transformation. Nonneoplastic mammalian cells in culture require a specific hormone(s) and growth factor(s) for initiation of DNA synthesis and mitosis, and an oncogene(s) product(s) may subvert normal growth regulatory mechanisms and lead to neoplastic transformation in culture. This paradigm was assessed in a serum-free bioassay with BALB/MK keratinocytes stably infected with retroviruses containing the "cytoplasmic" oncogenes v-H-ras, v-Ki-ras, v-erbB and v-fms (which encode growth factor receptors), v-fgr and v-mos. Viral transformants grew in serum-free medium containing insulin without epidermal growth factor (EGF), and the v-fgr oncogene abrogated both insulin and EGF requirements. Preliminary studies with v-myc, a "nuclear" oncogene, suggest this gene acts downstream from the insulin/IGF-1 and EGF pathways.

Finally, a novel keratinocyte growth factor (KGF) was isolated from serum-free medium conditioned by normal human embryonic fibroblasts. The protein was purified and characterized, and its biologic activity assessed by thymidine incorporation and proliferation assays done in defined medium. Like the FGF family and "cytoplasmic" oncogenes, KGF complements the insulin/IGF-1 signal transduction pathway.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

W. G. Taylor	Biologist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
J. S. Rubin	Biotechnology Fellow	LCMB	NCI

Objectives:

A fundamental event in carcinogenesis is subversion of normal growth regulation. A current model proposes that certain growth factors and membrane-associated receptors are encoded by v-*onc* genes and have structural or functional homology with normal cellular proteins which function in a normal mitogenic signal transduction pathway(s). Constitutive expression at an inappropriate point in the cell cycle, overexpression, or synthesis of an altered gene product may exert a sustained mitotic stimulus either on cells producing the mitogen(s) (autocrine) or on neighboring cells (paracrine). Interaction(s) between mitogens and/or receptors and the mitogenic pathways of normal and tumorigenic cells are of both theoretical and practical interest, either as a molecular model of growth regulation or a bioassay system in which strategies for intervention can be tested.

Mutually inclusive objectives include (a) establishment of a serum-free bioassay with which to compare the impact of known growth factors with that of viral oncogenes (v-*onc*) and v-*onc* gene products in the mitotic pathways of prototype epithelial cells, and (b) to probe for undefined mitogenic activity secreted by low passage stromal cells. In addition to an existing BALB/MK model system a battery of indicator cells was developed to define target cell specificity and aid in detection and classification of mitogens.

Methods Employed:

A sensitive serum-free clonal growth assay with BALB/MK keratinocytes is used to quantify responses to known mitogens, v-*onc* gene products or undefined substances with unknown specific activities produced by stromal tissue. This assay system permits the extended incubation period sometimes required to fully discriminate between an additive and synergistic response. Petri dishes are precoated with poly-D-lysine and fibronectin to aid adhesion and spreading, and commercially available hormones and growth factors are used to supplement serum-free medium. Gene complementation is assessed by systematically omitting growth factor(s). Endpoints for these assays are comparative increase in cell number or tritiated thymidine incorporation. Human embryonic fibroblasts (M426) or stromal cells derived from human tumor samples are grown to confluence in serum-free medium conditioned (SFCM) by the monlayer, and the activity of novel growth factors in the SFCM tests with quiescent populations of prototype cells is determined.

Major Findings:

The BALB/MK keratinocytes exhibit a critical requirement for epithelial cell growth factor (EGF) serum-containing medium. A chemically defined, serum-free medium was developed to assess more rigorously the minimum growth factor requirements of BALB/MK cells and investigate whether v-*onc* genes (or their gene products) alter the requirements for sustained proliferation. At a low

population density a combination of EGF and insulin, the latter apparently acting through the insulin and IGF-1 receptors, maintained a growth rate essentially identical to that in medium-containing serum and EGF. This observation is consistent with earlier reports that epithelial cell growth may be enhanced by reduction of serum concentration. Both acidic and basic fibroblast growth factors (aFGF and bFGF, respectively) substitute for EGF, but not insulin proliferation and incorporation assays. Infection of BALB/MK keratinocytes with retroviruses containing "cytoplasmic" oncogenes also permitted growth in defined medium-containing insulin but without EGF. Those tested were *ras* (v-H-*ras* and v-Ki-*ras*), v-*erb*B, v-*fms* (oncogenes which encode growth factor receptors), v-*fgr* and v-*mos*. The impact of the v-*fgr* oncogene, a member of the *src* subfamily, was unique as it conferred independence from both exogenous insulin and EGF. In separate studies, no evidence of a novel, secreted mitogenic activity for nontransformed BALB/MK cells was detected in SFCM generated by monolayers of the *fgr* transformant. Subsequently, a "nuclear" oncogene, v-*myc*, was introduced into BALB/MK cells to determine if a gene product putatively localized to the cell nucleus would also abrogate growth factor requirements in serum-free medium. To date, no differences in response to known growth factors is apparent between the progenitor BALB/MK and the *myc* transformants.

Finally, a novel growth factor for keratinocytes was isolated from serum-free culture medium conditioned by line M426, a normal human embryonic fibroblast. The factor, provisionally named keratinocyte growth factor (KGF), was purified by ultrafiltration and chromatography (see Z01CP05511-03). KGF exhibits an epithelial cell specificity in that it is strongly mitogenic for BALB/MK keratinocytes and induces elevated thymidine incorporation in human mammary as well as rhesus bronchial epithelia, but in contrast elicits little response from mouse or human fibroblasts. Like aFGF, bFGF and the "cytoplasmic" oncogenes tested to date, in chemically defined medium at low cell density KGF complements the insulin/IGF-1 signal transduction pathway. Efforts continue to determine if morphologically normal mesenchymal cells isolated from human tumor tissue secreted undefined factors which exert a subtle, chronic mitogenic stimulus for neighboring epithelium, since this might represent a rich source of novel, clinically relevant mitogens.

Publications:

Aaronson SA, Falco JP, Taylor WG, Cech AC, Marchese C, Finch PW, Rubin J, Weissman BE, Di Fiore PP. Pathways in which growth factors and oncogenes interact in epithelial cell mitogenic signal transduction. Ann NY Acad Sci (In Press)

Falco JP, Taylor WG, Di Fiore PP, Weissman BE, Aaronson SA. Interactions of growth factors and retroviral oncogenes with mitogenic signal transduction pathways of BALB/MK keratoinocytes. Oncogene 1988;2:573-8.

Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaronson SA. Identification and characterization of a novel growth factor specific for epithelial cells. Proc Natl Acad Sci USA 1989;86:802-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05469-04 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Identification of New Tyrosine Kinase Oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	G. Kruh	Medical Staff Fellow	LCMB	NCI
Others:	P. Perego	Guest Researcher	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have previously described the initial characterization of a novel human gene closely related to the abl oncogene. In order to understand the function of this gene, termed arg, we have now identified and characterized c-DNA clones of its transcript. The overall structure of the arg transcript is unusual in that the coding sequence is located at the 5' extremity, and at least 5.5 kb of the noncoding sequence is located at the 3' end. The complete coding sequence of the gene has been deduced from the large open reading frame located at the 5' of the transcript. Comparison of its amino acid structure with that of abl has revealed several interesting differences and similarities. Of note is the finding that the arg transcript is composed of alternative first exons that allow the translation of two arg proteins that differ only at their N-termini. This was a particularly surprising finding because only one arg transcript can be identified in northern blot analysis.

Clones containing the complete arg coding sequence have been assembled in appropriate mammalian expression vectors. Additionally a gag-arg construct analagous to v-abl has been engineered. These clones will allow an initial investigation of the biology of arg using the NIH/3T3 focus forming assay system. In addition to information pertaining to the transforming capability of arg, cell lines containing the normal arg expression vectors will allow the determination of the subcellular localization of the arg protein. Arg expression vectors will also be introduced into hematopoietic cells to determine if arg has the lymphocytic specificity that v-abl displays. In addition to the in vitro characterization of arg, viruses derived from the cell lines described above will be introduced into mice. These experiments will allow the in vivo tumorigenic properties of arg to be evaluated.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

G. Kruh	Medical Staff Fellow	LCMB	NCI
R. Perego	Guest Researcher	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI

Objectives:

To characterize a new oncogene and understand its role in human neoplasia.

Methods Employed:

cDNA clones of the *arg* oncogene are identified in cDNA libraries by standard method. cDNA clones are characterized using the techniques of restriction enzyme analysis and Southern blotting. Nucleotide sequence analysis of the cDNA clones is accomplished by the dideoxy chain termination method. *Arg* expression is studied using the techniques of RNA extraction and northern blot analysis.

Major Findings:

Overlapping *arg* cDNA clones have been fully characterized and the *arg* coding sequence has been deduced. This has allowed a revealing comparison with the *abl* oncogene, and has helped define the *abl*-subfamily of cytoplasmic tyrosine kinase genes. Mammalian expression vectors containing normal and activated forms of *arg* have been engineered.

Patents:

Kruh GD, Aaronson SA. US Patent (Pending): Definition of a Human Gene Related to but Distinct from the Abelson Proto-oncogene.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05472-04 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structural Characterization of Putative Growth Factor Receptor Gene c-erbB-2

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	O. Segatto	Visiting Associate	LCMB	NCI
Others:	S. A. Aaronson	Chief	LCMB	NCI
	P. P. Di Fiore	Visiting Scientist	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	1.0

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☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have shown that the erbB-2 product is constitutively phosphorylated in vivo on tyrosine residues. This reflects erbB-2 gp185 autophosphorylation and appears to correlate with the overall level of erbB-2 tyrosine kinase activity. By deletion analysis and site-directed mutagenesis, we have mapped the major sites of erbB-2 gp185 autophosphorylation in its COOH terminus. Deletion of the COOH terminus causes a 40-fold reduction of transforming activity. This suggests that the erbB-2 COOH terminus, while not essential for receptor signalling, exerts a positive regulatory role on the receptor catalytic activity. Tyrosine autophosphorylation does not seem to account entirely for this regulatory function since mutants generated by multiple simultaneous Tyr->Phe substitutions at the sites of autophosphorylation show only a fivefold reduction of transforming activity. It is therefore likely that other structural determinants of the erbB-2 COOH terminus further contribute to this regulatory activity, as also suggested by chimeric molecules betwen the epidermal growth factor receptor (EGFR) and the erbB-2 product. Further mutational analysis is centered around discrete regions of amino acid sequence divergence between the EGFR and erbB-2 gp185 catalytic domains, with the aim of defining the region(s) of both receptors involved in the recognition of cellular substrates.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

O. Segatto	Visiting Associate	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
P. P. Di Fiore	Visiting Scientist	LCMB	NCI

Objectives:

To study the mechanisms of signal transduction of the growth factor receptors *erb*B-2 and epidermal growth factor receptor (EGFR). Studies are directed toward assessing the structural domains of the mature gene products involved in signal transduction, catalytic activity and regulation of catalytic activity.

Methods Employed:

By means of site-directed mutagenesis techniques, we have generated and then expressed a series of *erb*B-2 and EGF receptor cDNA mutants in order to study the regulation of their catalytic activity as well as the biochemical pathways responsible for the different biologic activities of the two receptors. The biological activity of the mutant proteins is correlated with biochemical parameters such as level of protein expression, in vitro and in vivo catalytic activity, protein turnover, phosphoinositide turnover and tyrosine phosphorylation of specific cellular proteins.

Major Findings:

Tyrosine kinase activity is strictly required for the biochemical and biological activities of the EGFR and *erb*B-2 product. The COOH termini of both receptors are not involved in the recognition of cellular substrates. Rather, they appear to play a regulatory role on the catalytic domain. Autophosphorylation on tyrosine residues located in the COOH terminus of the *erb*B-2 product is part of this regulatory mechanism. Ongoing studies are directed at defining, within the catalytic domain of the two receptors, the structures involved in the differential recognition of cellular substrates.

Publications:

Segatto O, King CR, Pierce JH, Di Fiore PP, Aaronson SA. Different structural alterations upregulate in vitro tyrosine kinase activity and transforming potency of the *erb*B-2 gene. Mol Cell Biol 1988;8:5570-4.

King CR, Di Fiore PP, Pierce JH, Segatto O, Kraus MH, Aaronson SA. Oncogenic potential of the *erb*B-2 gene: frequent overexpression in human mammary adenocarcinomas and induction of transformation in vitro. In: Lippman ME, ed. Growth regulation of cancer. Proceedings of UCLA symposium. New York: Alan R. Liss, 1988;189-99.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05514-03 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Analysis of PDGF Receptor Role in Neoplastic Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	T. Matsui	Visiting Fellow	LCMB	NCI
	J. H. Pierce	Research Microbiologist	LCMB	NCI
	T. P. Fleming	IRTA Fellow	LCMB	NCI
	M. Ruggiero	Visiting Scientist	LCMB	NCI
	M. A. Heidaran	IRTA Fellow	LCMB	NCI
	W. J. LaRochelle	Guest Researcher	LCMB	NCI
	T. Miki	Visiting Scientist	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	0.0

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We identified a genomic sequence and cloned the cDNA of a novel receptor-like gene of the platelet-derived growth factor (PDGF) receptor colony-stimulating factor-1 (CSF-1) receptor subfamily. The gene recognized a 6.4-kb transcript that was coexpressed in normal human tissues with the 5.3-kb PDGF receptor mRNA. The expression of its cDNA in COS-1 cells led to the specific binding by 125 I-human PDGF which was competed by all three PDGF isoforms. Expression of the known PDGF receptor cDNA in COS-1 cells led to PDGF binding with a distinct pattern of competition by the same PDGF isoforms. The existence of genes encoding two PDGF receptors that interact in a distinct manner with three different PDGF isoforms likely confers considerable regulatory flexibility in the functional response to PDGF.

In order to investigate the functional responses specific to each receptor, we undertook efforts to express their cDNAs in cells normally devoid of either receptor. We demonstrated both that receptors can function in such cells and that each independently mediates major known PDGF activities including mitogenic signal transduction, chemotaxis and stimulation of phosphoinositide turnover. Their binding by different PDGF isoforms distinguishes the two receptor gene products functionally and establishes the newly identified alpha PDGF receptor as the preferred for human PDGF.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
T. Matsui	Visiting Fellow	LCMB	NCI
J. H. Pierce	Research Microbiologist	LCMB	NCI
T. P. Fleming	IRTA Fellow	LCMB	NCI
M. Ruggiero	Visiting Scientist	LCMB	NCI
M. A. Heidaran	IRTA Fellow	LCMB	NCI
W. J. LaRochelle	Guest Researcher	LCMB	NCI
T. Miki	Visiting Scientist	LCMB	NCI

Objectives:

To extend our knowledge of growth factor receptors as human proto-oncogenes, we have cloned and characterized a novel receptor-like gene of the PDGF receptor/CSF-1 receptor subfamily. To investigate the functional responses specific to each receptor gene, we have expressed their cDNAs in cells normally devoid of either receptor.

Methods Employed:

By using retroviral expression vectors, we have introduced the cDNAs in several cell lines which originally lacked receptor expression. We examined the PDGF activities including mitogenic signal transduction, chemotaxis stimulation of phosphoinositide turnover and cytoplasmic ionized calcium concentration in those transfectants. To examine the functional involvement of the PDGF receptor in neoplastic transformation, we developed the specific antibodies for either receptor and screened the internally activated receptors in human tumor cell lines.

Major Findings:

1. The cDNA expression of the newly isolated receptor-like gene in COS-1 cells led to specific binding by ^{125}I-human PDGF.

2. Expression of the known PDGF receptor cDNA led to PDGF binding with a distinct pattern of competition by three PDGF isoforms.

3. Both receptor cDNAs could function in 32D mouse hematopoietic cells normally devoid of either receptor using eukaryotic expression vectors.

4. Each receptor independently mediated major PDGF activities including mitogenic signal transduction, chemotaxis and phosphoinositide turnover.

5. The newly identified α PDGF receptor was the preferred receptor for human PDGF.

6. Intracellular as well as cell surface forms of two PDGF receptor gene products were tyrosine phosphorylated in v-*sis* transformants.

7. Activated PDGF receptors could be detected in human tumor cell lines which also express the PDGF-A or B chain.

Publications:

Matsui T, Heidaran M, Miki T, Popescu N, La Rochelle W, Kraus M, Pierce J, Aaronson SA. Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. Science 1989;243:800-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05546-02 LCMB

PERIOD COVERED: October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structural and Functional Characterization of v-sis Gene Product

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. A. Aaronson	Chief	LCMB NCI
Others:	N. A. Giese	IRTA Fellow	LCMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Platelet-derived growth factor (PDGF) is composed of two polypeptide chains encoded by separate genes, PDGF-A and PDGF-B. The oncogene of simian sarcoma virus, v-sis, is derived from the PDGF-B gene and its encoded transforming protein is a PDGF-B-like homodimer. Site-directed mutagenesis has defined an internal 84-codon region which mediates transforming activity. Further mutagenesis within this minimum transforming domain has revealed smaller domains of functional importance which also provide epitopes for binding and neutralization by a monoclonal antibody, designated anti-sis 1.

A complementary approach to identifying the PDGF-B receptor binding domain(s) has been accomplished by making PDGF-A/PDGF-B chimeras. PDGF-A and PDGF-B homodimers have distinct binding phenotypes. Characterization of chimeric proteins has allowed us to identify the region within PDGF-B responsible for its particular receptor-binding properties. Continuing studies on this project will be directed at further characterization of the receptor-binding domain and the identification of a competitive antagonist which will be useful in blocking PDGF-mediated responses.

To facilitate more detailed analysis of PDGF proteins, a baculovirus vector system was employed for the overexpression of PDGF-B. The processing and biological properties of recombinant PDGF-B were shown to be very similar to those of PDGF-B synthesized in mammalian cells. Also, recombinant PDGF-B was purified to near homogeneity using immunoaffinity chromatography, further demonstrating the utility of this system.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
N. A. Giese	IRTA Fellow	LCMB	NCI
W. J. LaRochelle	Guest Researcher	LCMB	NCI

Objectives:

The structural and functional characterization of the platelet-derived growth factor (PDGF) ligands.

Methods Employed:

Standard recombinant DNA techniques; DNA sequencing; oligonucleotide-directed mutagenesis; transfection assay; tissue culture; immunoprecipitation and SDS-PAGE analysis of proteins; COS cell assay for transient overexpression of proteins; baculovirus vector system for protein overexpression and analysis of PDGF receptor activation.

Major Findings:

1. Introduction of mutations throughout the minimum transforming domain of v-*sis*/PDGF-B and identification of smaller regions of structural and functional importance.

2. Mapping of an epitope required for binding of a neutralizing monoclonal antibody raised against PDGF-B.

3. Identification of a PDGF-B domain responsible for B-type PDGF receptor activation.

4. Overexpression, characterization of processing and biological properties and purification of v-*sis* protein produced using a baculovirus expression system.

Publications:

Giese N, May-Siroff M, LaRochelle WJ, Van Cyke Coelingh K, Aaronson SA. Expression and purification of biologically active v-*sis*/PDGF-B protein using a baculovirus vector system. J Virol (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05547-02 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Role of PDGF Expression in the Neoplastic Process

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M. Pech	Visiting Scientist	LCMB NCI
Others:	S. A. Aaronson	Chief	LCMB NCI

COOPERATING UNITS (if any)

Children's Hospital Medical Center, Cincinnati, OH (G. Jones)

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Gene Structure Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The v-sis oncogene of simian sarcoma virus (SSV) encodes a protein which is closely related to the human PDGF-B or -2 chain. The c-sis/platelet-derived growth factor (PDGF)-B gene, the human homologue for v-sis, has been cloned and characterized. When this gene is expressed under the control of a retrovirus promoter and introduced in NIH 3T3 cells in culture, it confers the transformed phenotype to these cells. The PDGF-B chain transcript has been observed in a variety of human glioblastomas and fibrosarcomas. In contrast, this transcript is not detectable in normal glial cells or fibroblasts. All of these findings along with the immunological demonstration of the PDGF-B product expressed by such human tumor cells support a role of PDGF-B in tumorigenesis. We have investigated the regulation of expression of this growth factor in vivo and in vitro. Overexpression of PDGF-B in vivo leads to formation of fibrosarcomas in mice. Low levels of expression of this human growth factor in different tissues was also achieved by introducing the gene into the germline of transgenic mice.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. Pech	Visiting Scientist	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI
S. R. Tronick	Chief, Gene Structure Section	LCMB	NCI

Objectives:

(1) To identify the regulator mechanisms governing the expression of the human PDGF-B (also referred to as PDGF-2) protein. (2) To characterize PDGF proteins in human tumor cells. (3) To determine which changes in the regulatory pathway are responsible for the aberrant expression of the PDGF-B protein in human malignancies. (4) To evaluate the role of PDGF-B expression in the generation of tumors in vivo. (5) To investigate the expression pattern of PDGF-B in vivo.

Methods Employed:

Standard recombinant DNA technology; cDNA cloning; nuclease S1 mapping and primer extension analysis of RNA; RNase A/T1 protection analysis of RNA; northern blots of RNA and Southern blots of DNA; DNA and RNA sequencing; deletion mutagenesis; site-specific mutagenesis using synthetic oligonucleotides; cellular transformation and animal tumorigenicity assays; electroporation of established and primary cell lines; enzymatic assays for the transient expression of reporter genes; gel retardation and DNase 1 footprint assays; standard techniques for rescue and propagation of retroviruses; generation of antisera directed against synthetic peptides; immunoprecipitation and immunoblotting; generation of transgenic mice.

Major Findings:

A retrovirus containing the entire human platelet-derived growth factor (PDGF)-B gene was constructed in order to investigate the in vivo biological activity of its encoded growth factor. When this virus was introduced into newborn mice, it reproducibly generated fibrosarcomas at the site of inoculation. Proviruses in each fibrosarcoma analyzed contained a deletion of 149 nucleotides downstream of the PDGF-B coding region. This deletion originated from an alternative or aberrant splice event which occurred within exon 7 of the PDGF-B gene and mimicked the *v-sis* oncogene. Thus, deletion of this region may be necessary for efficient retrovirus replication or for more potent transforming function. Evidence that the normal growth factor coding sequence was unaltered derived from RNase protection studies and inmmunoprecipitation analysis. Tumors were generally polyclonal but demonstrated clonal subpopulations. Moreover, tumor-derived cell lines became monoclonal within a few tissue culture passages and rapidly formed tumors in vivo. These findings argue that overexpression of the normal human PDGF-B gene product under retrovirus control can induce the fully malignant phenotype.

We investigated the regulation of PDGF-B mRNA expression during megakaryocytic differentiation of K562 cells. Induction by 12-O-tetradecanoylphorbol-13-acetate (TPA) led to an increase greater than 200-fold in PDGF-B transcript levels in these cells. Induction was dependent on protein synthesis and was

not enhanced by cycloheximide exposure. In uninduced K562 cells a minimal promoter region, which included sequences extending only 42 bp upstream of the TATA signal, was found to be as efficient as 4 kbp upstream of the TATA signal in driving expression of a reporter gene. We also functionally identified different regulatory sequence elements of the PDGF-B promoter in TPA-induced K562 cells. One region acted as a transcriptional silencer, while another region was necessary for maximal activity of the promoter in megakaryoblasts. This region was shown to bind nuclear factors and was the target for trans-activation in normal and tumor cells. In one tumor cell line, which expressed high PDGF-B mRNA levels, the presence of the positive regulatory region resulted in a 30-fold increase in promoter activity. However, the ability of the minimal PDGF-B promoter to drive reporter gene expression in uninduced K562 cells and normal fibroblasts, which contained no detectable PDGF-B transcripts, implies the existence of other negative control mechanisms beyond the regulation of promoter activity.

The different PDGF-B promoter deletion constructs were introduced into the germline of transgenic mice. The reporter gene activity was analyzed in different tissues and found mainly to be localized in brain and tail extract. A further analysis of those transgenic mice lines is under way.

In addition, we were able to express PDGF-B under the control of the mouse metallothionein-promoter in transgenic mice. The phenotype of those mice is now being studied under conditions were the expression of PDGF-B can be induced.

Publications:

Pech M, Gazit A, Arnstein P, Aaronson SA. Generation of fibrosarcomas in vivo by a retrovirus which expresses the normal PDGF-B chain and mimics the alternative splice pattern of the v-*sis* oncogene. Proc Natl Acad Sci USA (In Press)

Pech M, Rao CD, Robbins KC, Aaronson SA. Functional identification of regulatory elements within the promoter region of platelet-derived growth factor 2. Mol Cell Biol 1989;9:396-405.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05548-02 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of Expression Cloning System for Oncogene cDNAs

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	T. Miki	Visiting Scientist	LCMB	NCI
Others:	M. Crescenzi	Visiting Fellow	LCMB	NCI
	T. P. Fleming	NRSA Fellow	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	1.0

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We are developing an efficient system for isolation of dominant as well as recessive oncogenes. In this system, cDNA libraries are constructed in eukaryotic expression vectors using RNAs extracted from transformants or tumors. The library DNA can then be used to transfect NIH/3T3 cells. cDNA clones will be recovered from resulting foci and their structures analyzed. We have already developed a high efficiency cDNA cloning system which can direct the orientation of inserts in eukaryotic expression vectors.

During this reporting period, refinements of the cDNA expression cloning system and attempts to clone a dominant oncogene were carried out. The vectors for construction of cDNA libraries have been modified to optimize the expression level of the cDNA insert. An improved plasmid rescue mechanism was incorporated for quick recovery of cDNA clones from eukaryotic cells. Moreover, an efficient mechanism to construct subtraction libraries was included in our system, especially for cloning of tumor suppressor gene cDNAs.

A cDNA library was constructed from the RNA of an NIH/3T3 transformant which had been obtained by transfection of DNA from a tumor of a B6C3F1 mouse. Several foci were obtained and are being analyzed.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. Miki	Visiting Scientist	LCMB	NCI
M. Crescenzi	Visiting Fellow	LCMB	NCI
T. P. Fleming	NRSA Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI

Objectives:

Recessive oncogene and certain novel dominant oncogene cDNAs are difficult to clone because of their size and because probes for their detection are not available. One approach toward cloning such oncogenes relies upon an efficient expression cloning system of cDNA. The use of expression cloning has been hampered by inefficient synthesis of long cDNAs and low efficiency of DNA-mediated gene transfer (DNA transfection). To overcome these problems, we have developed a high efficiency cDNA cloning system and expression vectors designed to facilitate the isolation of novel oncogenes and other recessive genes which contribute to the malignant process. We are attempting to clone novel oncogene cDNAs using this system.

Methods Employed:

Standard molecular cloning methods were used to construct vectors. DNA-mediated gene transfer (DNA transfection) was used to introduce DNA into NIH/3T3 cells. RNA extraction and cDNA synthesis methods were used to construct cDNA libraries.

Major Findings:

1. The vectors for construction of cDNA libraries have been modified to express cDNA inserts at high levels. The H-*ras* oncogene was used as a "reporter gene." The LTR promoter taken from pZIPneo SV(x) showed efficient focus formation and a high level of protein synthesis, although the mRNA level was somewhat lower than other promoters.

2. Some additional restriction sites for infrequent cutters were added to excise the plasmid part from the lambda-plasmid composite vector. The plasmid replication origin has been replaced by the one with a higher copy number, and an ampicillin-resistant gene was added to the vector. These features were designed to facilitate the recovery of cDNA clones from eukaryotic cells.

3. In addition to the expression cloning system, differential screening of subtraction libraries would be another important approach toward cloning of tumor suppressor genes. Our vectors have been improved to construct subtraction libraries and to generate probes for differential hybridization.

4. We have detected activated oncogenes in spontaneously occurring or chemically induced benign and malignant hepatocellular tumors of the B6C3F1 mouse strain, the majority of which were identified as the activated forms of *ras* or *raf* genes. However, some appeared to be novel. We have started to clone the cDNAs of genes using our expression cloning

system. A cDNA library was constructed from the RNA of one of the transformants. The library DNA was used to transfect NIH/3T3 cells. Of 11 foci obtained, one showed G-418 resistance which is the selectable marker of our vector. Genomic DNA was extracted from the cells, and cDNA inserts were amplified by polymerase chain reaction (PCR). At least six plasmids were detected. We are attempting to recover the cDNA clones from the genomic DNA.

Publications:

Katamine S, Notario V, Rao CD, Miki T, Cheah MSC, Tronick SR, Robbins KC. Primary structure of the human *fgr* proto-oncogene product $p55^{c\text{-}fgr}$. Mol Cell Biol 1988;8:259-66.

Matsui T, Heidaran M, Miki T, Popescu N, La Rochelle W, Kraus M., Pierce J, Aaronson SA. Isolation of a novel receptor cDNA establishes the existence of two PDGF receptor genes. Science 1989;243:800-4.

Miki T, Matsui T, Heidaran MA, Aaronson SA. Efficient directional cloning system to construct cDNA libraries containing full-length inserts at high frequency. Gene (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05549-02 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Protein Kinases in Growth Factor-mediated Cell Activation and Transformation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S.A. Aaronson	Chief	LCMB	NCI
Others:	C.J. Molloy	Biotechnology Fellow	LCMB	NCI
	D. Bottaro	IRTA Fellow	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Protein phosphorylation constitutes a major mechanism through which growth factors and related transforming oncogenes influence intracellular events. In an effort to understand the role of specific protein phosphorylations in growth factor-mediated cell activation, experiments were performed using both epithelial and fibroblast cell lines expressing the epidermal growth factor (EGF)-receptor tyrosine kinase. In BALB/MK keratinocytes, EGF-receptor activation was accompanied by a phospholipase C-mediated phosphoinositide catabolism, resulting in the generation of the intracellular second messengers, inositol (1,4,5) trisphosphate and diacylglycerol. This led to the activation of protein kinase C, which was indicated by increased phosphorylation of the specific endogenous substrate protein p80. Further experiments, designed to identify specific tyrosine phosphorylated polypeptide substrates of the EGF-receptor kinase which may be involved in the mitogenic actions of EGF were performed using both one- and two-dimensional gel electrophoresis coupled to immunoblotting with specific antiphosphotyrosine antibodies. The results showed that tyrosine phosphorylation of several proteins was rapidly induced following EGF stimulation of fibroblasts expressing the EGF-receptor. These included proteins of apparent molecular weights of 36(pI 6.5), 40(pI 6.0), 42(pI 5.8), 70(pI 4.8), 80(pI 5.3), 120(pI 5.0) and 150(pI 5.4) kd. Similar experiments were carried out following platelet-derived growth factor (PDGF) stimulation in an attempt to identify common pathways of mitogenic signal transduction requiring tyrosine-specific phosphorylation. Preliminary results indicate that both EGF and PDGF rapidly induce tyrosine-specific phosphorylation of overlapping subsets of proteins, suggesting that some of these molecules may have important roles in growth factor-mediated signal transduction and transformation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S.A. Aaronson	Chief	LCMB	NCI
C.J. Molloy	Biotechnology Fellow	LCMB	NCI
D. Bottaro	IRTA Fellow	LCMB	NCI

Objectives:

To study the role of protein phosphorylation in growth factor-mediated cell activation and oncogene transformation.

To identify important endogenous polypeptide substrates of transforming and signal transducing protein tyrosine kinases.

Methods Employed:

Standard cell culture techniques, including metabolic radiolabeling of cell proteins, protein extraction techniques, one- and two-dimensional polyacrylamide gel electrophoresis, protein immunoblotting and immunoprecipitation assays, standard in vitro enzyme assays, affinity chromatography of polypeptides using immobilized lectins and antibodies, and standard antibody production.

Major Findings:

In an effort to understand the role of specific protein phosphorylation events in growth factor mediated cell activation and transformation, experiments were performed using both epithelial and fibroblast cell lines expressing the EGF-receptor tyrosine kinase. Tyrosine protein kinase activity elicited by EGF, which was manifested as receptor autophosphorylation as well as endogenous substrate phosphorylation, was characterized by immunoblotting using specific antiphosphotyrosine antibodies. In the EGF-responsive BALB/MK keratinocyte cells, EGF-receptor activation was accompanied by a phospholipase C-mediated phosphoinositide catabolism, resulting in the generation of the second messengers inositol (1,4,5) trisphosphate and diacylglycerol. This led to the activation of protein kinase C, which was indicated by increased phosphorylation of the specific endogenous substrate protein for this kinase, p80. Similarly, in experiments using NIH3T3 fibroblasts overexpressing the EGF-receptor, EGF caused a pronounced stimulation of phosphoinositide metabolism, resulting in the activation of protein kinase C. These results implicate this biochemical pathway in at least some of EGF's actions, and suggest that enzymes participating in this pathway may be relevant targets of the activated EGF-receptor tyrosine kinase.

Studies designed to identify specific tyrosine-phosphorylated polypeptides involved in growth factor signalling were performed. Utilizing one- and two-dimensional polyacrylamide gel electrophoresis coupled with protein immunoblotting using antiphosphotyrosine antibodies, several potential substrates of the activated EGF-receptor kinase were identified in fibroblasts overexpressing the EGF-receptor. These included proteins of apparent molecular weights of 36(pI~6.5), 40(pI~6.0), 42(pI~5.8), 70(pI~4.8), 80(pI~5.3), 120(pI~5.0) and 150(pI~5.4) kd. Similar experiments were carried out using PDGF-stimulation of the same cells in an attempt to identify common pathways of mitogenic signal transduction requiring tyrosine-specific

phosphorylation. Preliminary results show that both EGF and PDGF rapidly induce tyrosine-specific phosphorylation of overlapping subsets of proteins, suggesting that some of these molecules may have important roles in growth factor-mediated signal transduction and transformation. Future studies will attempt to purify and further characterize these tyrosine phosphorylated protein targets with the goal of identifying their specific function in cell growth and transformation.

Publications:

Di Marco E, Pierce JH, Fleming TP, Kraus M, Molloy CJ, Aaronson SA, Di Fiore PP. The interaction between TGF and EGF-receptor: relevance for the transformed phenotype in vitro and in vivo. Oncogene (In Press)

Moscat J, Fleming TP, Molloy CJ, Aaronson SA. The calcium signal for BALB/MK keratinocyte terminal differentiation induces sustained alterations in phosphoinositide metabolism without detectable protein kinase C activation. J Biol Chem (In Press)

Moscat J, Molloy CJ, Fleming TP, Aaronson SA. Epidermal growth factor activates phosphoinositide turnover and protein kinase C in BALB/MK keratinocytes. Mol Endocrinol 1988;9:799-805.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05596-01 LCMB

PERIOD COVERED: October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Purification and Characterization of Epithelial Cell Mitogens

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	J. S. Rubin	Biotechnology Fellow	LCMB	NCI
	D. Bottaro	IRTA Fellow	LCMB	NCI
	P. W. Finch	Visiting Fellow	LCMB	NCI
	D. Ron	Visiting Associate	LCMB	NCI
	A. Chan	Visiting Fellow	LCMB	NCI
	J. Wong	Howard Hughes Fellow	LCMB	NCI
	D. Morris	NCI SRT Fellow	LCMB	NCI
	W. G. Taylor	Biologist	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH: Laboratory of Cellular and Molecular Biology

SECTION: Molecular Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Studies have focused on defining the pattern of expression, structure-function relationships and receptor interactions of the recently purified and cloned mitogen, keratinocyte growth factor (KGF, formerly termed epithelial cell growth factor I).

Another protein, originally identified as a second epithelial cell growth factor (EpGF II), was proven not to be responsible for this mitogenic activity. The isolated molecule binds tightly to heparin and is expressed in a variety of tissues, but presently has no known function.

Renewed attempts to purify the true mitogen have resulted in the identification of proteins of about 90 kilodaltons and 60 kilodaltons as the most likely candidates. Electroelution of these protein bands from SDS-polyacrylamide gels, followed by proteolytic cleavage and microsequence analysis are now underway.

A highly enriched preparation of this growth factor activity triggered tyrosine phosphorylation in mitogenically responsive cells. Preliminary experiments suggest that the major labeled protein may correspond to the growth factor receptor.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB, NCI
J. S. Rubin	Biotechnology Fellow	LCMB NCI
D. Bottaro	IRTA Fellow	LCMB NCI
P. W. Finch	Visiting Fellow	LCMB NCI
D. Ron	Visiting Associate	LCMB NCI
A. Chan	Visiting Fellow	LCMB NCI
J. Wong	Howard Hughes Fellow	LCMB NCI
D. Morris	NCI SRT Fellow	LCMB NCI
W. G. Taylor	Biologist	LCMB NCI

Objectives:

To purify and study growth regulatory factors that stimulate or inhibit the proliferation of epithelial cells; to determine whether the production of such factors influences the incidence of human malignancy or other pathological states characterized by altered patterns of proliferation.

Methods Employed:

Conventional column chromatography as well as high performance liquid chromatography and fast protein liquid chromatography; electroelution from SDS-polyacrylamide gels; ^{3}H-thymidine mitogenesis bioassay; metabolic labeling with ^{35}S-methionine and -cysteine, as well as P^{32}-orthophosphate; enzyme-linked immunosorbent assay; western blot and northern blot analysis; iodination of proteins.

Major Findings:

1. Keratinocyte growth factor (KGF) is expressed in the skin, gastrointestinal tract, kidney and lung as well as in stromal cell lines established from epithelial tissues.

2. Recombinant KGF prepared with a bacterial expression system is biologically active and has properties similar to the naturally occurring protein.

3. Chimeras containing portions of KGF and acidic fibroblast growth factor (aFGF) have mitogenic activity and heparin-binding properties that help define structure-function relationships which govern the native molecules.

4. KGF stimulates tyrosine kinase activity in BALB/MK cells, with preliminary evidence of phosphorylation of a putative receptor as well as other substrates.

5. KGF has high affinity binding sites in BALB/MK cells but not on NIH/3T3 cells, consistent with its target cell specificity.

6. The previous identification of a second epithelial cell growth factor (formerly termed EpGF II and now simply EpGF) was mistaken, but correct identification has been narrowed to two or three possibilities which are being further characterized.

7. A highly enriched preparation of EpGF stimulates tyrosine kinase activity in the human mammary epithelial cell line, B5/589, with a single major substrate (EpGF receptor?) migrating as a 160 kilodalton protein.

Publications:

Aaronson SA, Falco JP, Taylor WG, Cech AC, Marchese C, Finch PW, Rubin J, Weissman BE, Di Fiore PP. Pathways in which growth factors and oncogenes interact in epithelial cell mitogenic signal transduction. In Galeotti T, Cittidini A, Mori G, Scarpa A, eds. Membrane in cancer cells. New York: Ann NY Acad Sci, 1988;320-36.

Blam SB, Mitchell R, Tischer E, Rubin JS, Silva M, Silver S, Fiddes JC, Abraham JA, Aaronson SA. Addition of growth hormone secretion signal to basic fibroblast growth factor results in cell transformation and secretion of aberrant forms of the protein. Oncogene 1988;3:129-36.

Finch PW, Rubin JS, Miki T, Aaronson SA. Human KGF is FGF-related with properties of a major paracrine effector of epithelial cell growth. Science (In Press)

Rubin JS, Osada H, Finch PW, Taylor WG, Rudikoff S, Aaronson SA. Purification and characterization of a newly identified growth factor specific for epithelial cells. Proc Natl Acad Sci USA 1989;86;802-6.

Patents:

Rubin JS, Finch PW, Aaronson SA. US Patent (Pending): DNA Encoding a Growth Factor Specific for Epithelial Cells.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05597-01 LCMB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Inositol Lipid Turnover in EGF-induced Cell Growth

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Ruggiero	Visiting Scientist	LCMB	NCI
Others:	J. H. Pierce	Research Microbiologist	LCMB	NCI
	T. P. Fleming	NRSA Fellow	LCMB	NCI
	S. A. Aaronson	Chief	LCMB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We studied the effect of epidermal growth factor (EGF) on signal transduction on three cell lines of different origin. A long terminal repeat (LTR)-driven expression vector containing the normal human EGF receptor was introduced into NIH/3T3, NR6 and 32D cell lines by transfection, leading to overexpression of the receptor. In the cell lines overexpressing the receptor, EGF stimulated the rapid formation on inositol polyphosphates, 1,2-diacylglycerol and arachidonic acid, the mobilization of intracellular Ca++ and the activation of protein kinase C. Formation of Ca++-mobilizing inositol (1,4,5)-trisphosphate was very rapid and transient, reaching its peak 15-30 seconds after stimulation. Conversely, the level of inositol (1,3,4)-trisphosphate increased more slowly, but remained elevated up to five minutes. Measurement of intracellular Ca++ concentration by fura 2, revealed both intracellular mobilization and influx from the outside in response to the growth factor. Taken together, these data indicate that the reconstituted human EGF receptor is able to couple to the phosphoinositide-related intracellular signalling machinery. Since EGF was fully mitogenic in the cell lines tested and induced a similar pattern of second messenger formation irrespective of the origin of the cell line, we suggest that inositol lipid and arachidonic acid metabolism might play a crucial role in the transduction of the EGF mitogenic signal.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

M. Ruggiero	Visiting Scientist	LCMB	NCI
J. H. Pierce	Research Microbiologist	LCMB	NCI
T. P. Fleming	NRSA Fellow	LCMB	NCI
S. A. Aaronson	Chief	LCMB	NCI

Objectives:

1. To characterize the effect of epidermal growth factor (EGF) on inositol lipid turnover, calcium metabolism, protein kinase C and arachidonic acid metabolism.

2. To define the role of inositol lipid turnover in EGF-induced cell proliferation.

Methods Employed:

The study of inositol lipid metabolism was carried out by analyzing inositol phosphate formation and polyphosphoinositide turnover. Water-soluble inositol polyphosphates were separated by ion-exchange chromatography and by high pressure liquid chromatography. Polyphosphoinositides were separated by thin-layer chromatography on silica gel plates. The study of arachidonic acid metabolism was performed by separating arachidonic acid and its metabolites on thin-layer chromatography plates. Diacylglycerol was also analyzed by thin-layer chromatography. Intracellular calcium concentration was assessed by the use of the fluorescent probe "fura 2." Protein kinase C activation was determined by monitoring the phosphorylation of intracellular protein substrates.

Major Findings:

We studied the effects of EGF on inositol lipid metabolism and DNA synthesis in an EGF receptor-overexpressing NIH/3T3 fibroblasts. In order to compare the effect of EGF with that of a mitogen known to stimulate inositol lipid turnover, we also measured [^{3}H]inositol phosphate accumulation and [^{3}H]thymidine incorporation in response to platelet-derived growth factor (PDGF) in the same cell line. The results obtained indicate that the ability of EGF and PDGF to stimulate phosphoinositide turnover correlated well with their mitogenic effect, strongly suggesting a relationship between inositol lipid metabolism and cell growth.

To determine whether the effect of EGF on inositol lipid metabolism was a phenomenon generalizable to other cell types, we measured the rapid (10 minutes) accumulation of radioactive inositol phosphates in wild-type and EGF receptor-overexpressing cell lines. A significant increase in inositol phosphate accumulation was detectable only in the lines overexpressing the receptor, whereas no effect was detected in the parental lines.

In order to further characterize the inositol trisphosphates formed in response to EGF, inositol (1,4,5)trisphosphate and inositol (1,3,4)trisphosphate were separated by high pressure liquid chromatography (HPLC) and identified by co-elution with reference standards. In all the cell

lines tested, inositol (1,4,5)trisphosphate was formed rapidly (15-30 seconds) and transiently, returning to basal level in about five minutes. Inositol (1,3,5)trisphosphate, the product of successive phosphorylation and dephosphorylation of the 1,4,5-isomer, showed a slower increase, although it remained elevated for up to five minutes. Inositol monophosphates continued to increase for the entire length of the experiment and, 10 minutes after EGF addition, they represented more than 90% of the total inositol phosphates formed.

Then we sought to determine whether chelation of extracellular Ca^{++} or inhibition of cyclooxygenase activity could impair the ability of the growth factor to stimulate phosphoinositide turnover. Neither chelation of extracellular Ca^{++} by ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) nor inhibition of prostaglandin synthesis by indomethacin, prevented EGF from stimulating inositol lipid turnover as monitored by inositol phosphate formation. However, under these conditions, inositol phospholipid turnover was significantly reduced. These results suggest that influx of extracellular Ca^{++} and prostaglandin formation may enhance EGF-stimulated inositol lipid metabolism by acting as auto-amplificative second messengers.

Accumulation of radioactive arachidonic acid was investigated in wild-type and EGF receptor-overexpressing cells, after prelabeling the cultures with [^{14}C]arachidonic acid. EGF induced the formation of diacylglycerol and the liberation of arachidonic acid from [^{14}C]arachidonic acid-labeled phospholipids in EGF receptor-expressing 32D cells and NR6 fibroblasts. Chelation of extracellular Ca^{++} by EGTA significantly decreased the extent of diacylglycerol and arachidonic acid formation in response to EGF in EGFR-32D cells.

We measured protein kinase C activation in response to EGF in EGF receptor-overexpressing fibroblasts (NIH/3T3 and NR6) by monitoring the phosphorylation of a specific endogenous substrate, an acidic 80-kd protein (p80). Our results indicate that EGF stimulation led to activation of protein kinase C in cells overexpressing the receptor.

Finally, we measured intracellular Ca^{++} mobilization in EGFR-32D cells. As with inositol lipid and arachidonic acid metabolism, EGF was found to have a marked effect on [Ca^{++}]intracellular in EGFR-32D, but not in the parental 32D line. When these studies were performed in the presence of five mM EGTA, the magnitude of the EGF-induced [Ca^{++}]intracellular peak was reduced. These results indicate that EGF increased [Ca^{++}]intracellular in EGFR-32D cells by a dual mechanism, inducing both mobilization from intracellular store(s) and influx from the extracellular medium.

In conclusion, our study demonstrates that the normal human EGF receptor. expressed at high level, is able to couple to inositol lipid and arachidonic acid metabolism in cell lines that are mitogenically stimulated by EGF. Therefore, the ability to trigger phosphoinositide and arachidonate metabolism appears to be characteristic of the receptor itself, independently of the origin of the cell line into which it was transfected.

Publications:

Pierce JH, Ruggiero M, Fleming TP, Di Fiore PP, Greenberger JS, Varticovski L, Schlessinger J, Rovera G, Aaronson SA. Signal transduction through the EGF receptor transfected in IL3-dependent hematopoietic cells. Science 1988;233:628-31.

Ruggiero M. Membrane receptor and signal transduction in tumour cells in "Membrane-linked diseases." In Ohnishi ST, Ohnishi T, eds. New York: Taylor and Francis Publishers (In Press)

Ruggiero M, Srivastava SK, Fleming TP, Ron D, Eva A. NIH/3T3 fibroblasts transformed by the *dbl* oncogene show altered expression of bradykinin receptors effect on inositol lipid turnover. Oncogene (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05598-01 LCMB

PERIOD COVERED: October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Autocrine Mechanism for v-sis Transformation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	S. A. Aaronson	Chief	LCMB	NCI
Others:	T. P. Fleming	Guest Researcher	LCMB	NCI
	K. C. Robbins	Chief, Cellular and Molecular Biology Section	LOBP	NIDR
	T. Matsui	Visiting Fellow	LCMB	NCI
	C. Molloy	Biotechnology Fellow	LCMB	NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cellular and Molecular Biology

SECTION

Molecular Biology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER
1.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Using v-sis-transformed fibroblast cell lines, we were able to demonstrate that both alpha and beta platelet-derived growth factor (PDGF) receptors are activated in an intracellular compartment, yet a surface localization is required for coupling the activated receptors to a mitogenic response. These findings served as a useful model system to investigate human tumor cell lines and understand the role PDGF isoforms may have in the neoplastic process. Additionally, our studies indicate that the drug suramin, which can completely abrogate the transformed phenotype in sis/3T3 transformed cell lines, is able to significantly alter the proliferation of many human tumor cell lines that express either alpha or beta PDGF receptors and the A or B chain of PDGF.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. A. Aaronson	Chief	LCMB	NCI
T. P. Fleming	Visiting Fellow	LCMB	NCI
K. C. Robbins	Chief, Molecular and Cellular Biology Section	LOBP	NIDR
T. Matsui	Visiting Fellow	LCMB	NCI
C. Molloy	Biotechnology Fellow	LCMB	NCI

Objectives:

1. To determine the cellular site of v-*sis*-induced transformation.

2. To determine whether the newly discovered alpha platelet-derived growth factor (αPDGF) receptor is activated in v-*sis* transformation.

3. To elucidate PDGF receptor activation in human tumor cell lines.

4. To observe the biologic effects of suramin in the transformed phenotype.

Methods Employed:

Western blotting analysis for identification of protein products and the determination of the phosphorylated forms; DNA transfection to isolate cell lines for analysis of transforming activity; DNA synthesis analysis of cells; chemically defined media growth analysis; recombinant DNA techniques for the isolation and amplification of genes; analysis of genetic structure using restriction endonuclease mapping nucleotide sequencing and cDNA cloning.

Major Findings:

V-*sis* represents a prototype for the class of oncogenes that encode growth factors. Whether its PDGF-like product functionally activates cognate receptors within the cell or at the cell surface has potential implications in efforts to intervene with the transformed phenotype. In our studies we have determined that intracellular as well as cell surface forms of two PDGF receptor gene products are tyrosine phosphorylated in v-*sis* transformants.

In a chemically defined medium in which cell growth was dependent on v-*sis* expression, proliferation was partially inhibited by PDGF neutralizing antibody but completely blocked by suramin. Suramin treatment resulted in a marked reduction in tyrosine phosphorylated cell surface α and β PDGF receptors but had no effect on the level of tyrosine phosphorylation of intracellular receptor species. These findings indicate that suramin action must be confined to the cell surface and that activated receptors localized internally are not capable of transducing the mitogenic signal.

Extending these studies to human tumor cell lines, both α and β PDGF receptor activation was observed in several glioblastomas and fibrosarcomas. That PDGF receptor tyrosine phosphorylation is evident in both the mature and immature forms indicates that an intracellular activation of the receptors occurs. Varying degrees of growth inhibition can be observed in these human tumor cell lines after suramin treatment. These findings have general implications

concerning localization within the cell of critical targets of growth factor receptor action as well as approaches toward intervention with autocrine-associated malignancies.

Publications:

Fleming TP, Matsui T, Molloy CT, Robbins KC, Aaronson SA. Autocrine mechanism for v-*sis* transformation requires cell surface localization of internally activated growth factor receptors. Proc Natl Acad Sci USA (In Press)

Kraus MH, Pierce JH, Fleming TP, Robbins KC, Di Fiore PP, Aaronson SA. Mechanisms by which genes encoding growth factors and growth factor receptors contribute to malignant transformation. In Galeotti T, Cittadini A, Ner G, Scarpa A, eds. Membranes in cancer cells. New York: Ann NY Acad Sci 1988;320-36.

Ruggiero M, Srivastava SK, Fleming TP, Ron D, Eva A. NIH/3T3 fibroblasts transformed by the *dbl* oncogene show altered expression of bradykinin receptors effect on inositol lipid turnover. Oncogene (In Press)

CONTRACT IN SUPPORT OF ALL LABORATORY PROJECTS:

STATE OF CALIFORNIA DEPARTMENT OF HEALTH SERVICES (N01-CP-51001-35)

Title: Breeding and Production of 129/J and NFR Mice and Specified Services

Current Annual Level: $154,655

Man Years: 3.25

Objectives: To provide in vivo support for four major research efforts within the LCMB: (1) viral and cellular genes involved in malignant transformation; direct effect of specific oncogenes introduced into appropriate animals by viral recombinants; (2) analysis of genetically altered target cells by grafting into immunodeficient athymic nude host mice; (3) the role of host immune response in oncogene-induced tumors; and (4) heterotransplantation of human tumor cell-derived lines in athymic nude mice.

Major Contributions: The purpose of this contract is to provide support services for research conducted by LCMB; therefore, a discussion of major contributions will be found in the projects conducted by LCMB.

Proposed Course: This contract has been negotiated to run from March 1, 1985 through September 30, 1989. A continuation of this contract (NCI-CP-NO-95618-36) will commence October 1, 1989 and terminate September 30, 1993.

ANNUAL REPORT OF

THE LABORATORY OF MOLECULAR ONCOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 through September 30, 1989

The Laboratory of Molecular Oncology (LMO) has pioneered in the studies elucidating the structural relationship between the oncogenes of transforming retroviruses and their cellular prototypes (proto-oncogenes). This work has typically been facilitated by structural comparisons at the nucleic acid and predicted protein levels. While this important approach is still continuing, it is becoming more imperative to understand the biological and functional relationships between the normal proto-oncogene and its malignantly transforming counterpart. In the LMO we have focused upon this determination of biological function by studying these genes and their action at two levels: a) by investigating the retrovirus and the cells infected by them; and b) by examining various defined cells and tissues for the expression of the homologous proto-oncogenes using the retroviral oncogenes as probes, in order to understand the mechanism of oncogene action and how it cooperates with other growth regulator molecules within the normal cell.

Murine Leukemia Virus

To study the mechanism and cooperative role of the myb and ets oncogenes of the avian erythroleukemia virus, E26, in oncogenesis and to determine the biological function of v-ets and its cellular homologues in altering cell growth and hematopoietic development, we developed novel retroviral vectors containing these oncogenes that would infect murine systems to provide new biological assays that would characterize these functions. A replication-defective murine retrovirus, ME26, was constructed in our Laboratory by insertion of the avian gag-myb-ets oncogene sequences, derived from the avian E26 leukemia virus, into an Abelson murine leukemia virus-derived retroviral vector. This novel virus induces myeloid and erythroid leukemias in newborn mice. Histological analysis of diseased organs indicated that the majority of these leukemias (54%) were erythroid and myeloid neoplasms, while the remainder were lymphoblastic leukemias similar to that observed with helper virus alone. We found that leukemic cells from early cases of hematopoietic disease, contained multiple copies of integrated ME26 provirus and there was evidence that at least some of the tumors were clonal in origin. We found also that ME26-infected or transfected NIH 3T3 cells form foci of mitogenically-stimulated, morphologically-altered cells in defined media at low serum levels, and can grow in serum-free media in the absence of added growth factors. Recently, we constructed a recombinant provirus containing only the v-ets-specific sequences; cells transfected with this construct were able to express the 60Kd v-ets protein recognized by the ets-specific antisera. Furthermore, transfected cells formed transformed foci in defined media, even at low serum concentrations, albeit at 10- to 20-fold lower efficiencies.

Removal of the 34 amino acids of p15-gag residues did not change the focus-forming efficiency, suggesting that the myristilation signal of the murine p15-gag has no effect in vitro. Comparison of the biological activity of v-ets with that of chicken c-ets-1 in tissue culture suggests that the 5' ets sequences are a critical region in the function of ets. In addition, different NIH 3T3 cell lines infected with ME26 show different morphologic responses, suggesting further that ME26 viral transforming function in these cells may involve serum factors and their ability to interact with specific cellular factors.

ovc Oncogene

To further the studies on the activation of oncogenes in viruses and human tumors, we have continued to analyze the DNA sequences associated with the ovc oncogene, a human transforming sequence activated during DNA transfection and derived from the human ovarian carcinoma cell line, OVCAR-3. We have mapped a portion of that gene to chromosome band 9p24 by in situ analysis, and have identified a restriction fragment length polymorphism using this probe that will be a useful genetic marker in segregation studies involving human chromosome 9. Sequence analysis of the entire 2.2kb chromosome 9-derived ovc fragment reveals no significant homology to any known genetic sequences. We have also detected a transfectable sequence in a spontaneously tumorigenic human cell line which induces tumorigenicity and serum-independent growth, but not morphological transformation, in NIH 3T3 mouse fibroblasts. Hybridization analysis with oncogene and growth factor probes suggests this represents a potentially new oncogene or growth factor sequence. Recently, we have constructed a hygromycin selectable retroviral vector which expresses activated human ras^H that can be used in conjunction with other selectable retroviral vectors. We are using this vector to study the effects of multiple oncogene expression in infected cells. We have shown that low levels of activated $p21^{ras}$ will not induce the tumorigenic phenotype in murine and human cells, suggesting that a threshold level of even an activated ras is required to transform susceptible cells.

Retroviral-specific Enzymes

To continue the characterization of retroviruses, we assayed a variety of endogenous enzyme activities, finding a novel DNA topoisomerase I (topo I) activity associated with two strains of purified human immunodeficiency virus type-1 (HIV-1), equine infectious anemia virus (EIAV) and Moloney murine leukemia virus (Mo-MuLV). This topo I activity was removed from the viral lysate by anti-topo I serum. Western blot analysis indicates that an 11.5 Kd protein from both strains of HIV-1 and an 11 Kd protein from EIAV is recognized by this anti-topo I serum. No similar protein can be detected in the appropriate nuclear or cytoplasmic cell extracts. Moreover, topo I activity was present in isolated and purified EIAV cores. Immunoprecipitation assay using anti-topo I serum and Western blot analysis using anti-p11 EIAV nucleocapsid protein serum indicates that the anti-topo I serum removed the p11 nucleocapsid protein from the EIAV cores, together with topo I activity. These results suggest that the topo I activity is associated with the p11 nucleocapsid protein found in the EIAV cores.

The ets Oncogenes

To further elucidate the relationships between the transforming oncogene and the normal homologs of the ets genes, we have determined the complete nucleotide sequence of the chicken, mouse and human ets-1 and ets-2 genes; comparing them to each other and to the v-ets gene of E26. We found the cellular ets genes form a family of genes related to the oncogene containing unique 5′ and 3′ sequences. These structural differences in the viral ets gene may be responsible for the oncogenic potential of this retrovirus. We have found that the human ETS-1 gene product is over 95% identical to the chicken ets-1 gene. By contrast, we have found the mammalian ets-2 genes in humans and mice encode nearly identical amino acids, and are over 90% conserved, relative to the chicken ets-1 gene. Functionally, the ets-2 gene appears to have mitogenic activity upon transfection into eukaryotic cells. Alignment of the predicted ets proteins has allowed us to formulate a model for the ets gene family, showing that three distinct domains exist. The domain closest to the carboxy-termini is highly conserved in all predicted gene products from species ranging from human to Drosophila. The domain located at the amino terminal end of the ets proteins is more divergent, being highly conserved only between the same gene isolated from different species (e.g., chicken ets-1 vs. human ETS-1; mouse ets-2 vs. human ETS-2). The central domain of the ets proteins is found to be most divergent, even between the cognate genes. Thus, ets represents a proto-oncogene family of genes whose members are diverging at variable rates. Recombinant DNA technology is being used to generate mutants to evaluate the function of these three domains. Our understanding of the regulation of the ets genes has also been facilitated by structural and functional analysis of their promoter region. We have determined the nucleotide sequence of the human ETS-2 oncogene promoter region. The transcription initiation sites were determined by various mapping methods. The presence of multiple initiation sites is consistent with the absence of typical TATA and CAAT "boxes" in the surrounding sequence. Several repeat regions, two consensus AP2 and three putative Sp1 binding sites can be identified in the promoter region. A GC element with dyad symmetry is seen next to the major initiation site proximal to an unusually long (~250 bp) polypurine polypyrimidine tract. This polypurine polypyrimidine tract is essential for the activity of the ETS-2 promoter. Fusions of a series of deletion fragments of the promoter with the CAT gene, and subsequent transfection into human cell lines indicates that the sequences from -3600 bp to -159 bp are responsible for the promoter function. Further truncation gradually reduces the activity of the promoter. A number of S1 hypersensitive sites have been identified proximal to the transcription initiation region near the cis regulatory elements of the ETS-2 promoter. The polypurine polypyrimidine tract, proximal to the promoter, can act as a transcriptional activator in a transfection assay when it is placed upstream of the α-globin gene promoter. The identified ETS-2 promoter will be used to isolate and characterize trans factors involved in the ETS-2 gene transcriptional regulation.

Proto-ets Gene Family

Cellular sequences homologous to the ets region of the chicken retrovirus, E26, have been found in Drosophila in this laboratory. The characterized

portion of this gene corresponds to the last two exons of the chicken c-ets-1 gene, and has over 90% homology at the predicted amino acid level. It is designated D-ets, is localized on chromosome 3R at position 58A/B, and produces a single transcript of 4.7kb in all developmental stages. Hybridization of the Drosophila cDNA library at lower stringency has led to the isolation of a cDNA clone that shows considerable homology to v-ets, but which is not D-ets. This gene, called D-elg for Drosophila ets-like gene, has ~60% homology with D-ets and is located on chromosome 3R at 97D. This new gene produces two transcripts of 2.3 and 2.0kb in embryo, pupae, and adult stages. A third gene, presently called EBB, shows a significant degree of homology by hybridization with v-ets. It, too, is located on chromosome 3 at position 66A. This gene is expressed as a 1.6kb RNA species in only the pupae and adult stages. Thus, Drosophila appears to have conserved the 3′ region of the ets gene in at least two different genes. Drosophila can provide a system to determine the function of these genes. To study the function of the Drosophila ets gene product, a chimeric sequence (human 5′ end and Drosophila 3′ end) in a P-element vector has been constructed. This chimera will be microinjected into embryos, where the production of mutant flies will begin to supply information on its developmental and biochemical function.

Previous studies have suggested that fish have oncogene sequences homologous to those found in mammalian and avian species. We were the first to confirm the presence of fish oncogenes by isolating and sequencing the c-myc gene from rainbow trout. In order to examine the role of fish oncogenes in fish tumors, we developed a transfection system in which fish DNA was extracted from (1) lymphosarcomas from feral Northern pike (Esox lucius), (2) hepatocellular carcinomas and adenomas from feral white perch (Morone americana), (3) chemically-induced hepatic tumors in Japanese medaka (Oryzias latipes). The transforming ability of fish tumor DNA was examined by standard focus assay, nude mouse assay, and colony selection assay. DNA from a diethylnitrosamine-induced cholangiocarcinoma in medaka was the most efficient in transformation of NIH 3T3 cells. Tertiary transfectants caused formation of tumors in nude mice of >20mm in one week following injection. Southern blot analysis of these transfectant DNAs showed specific bands homologous to highly repetitive fish DNA present only in tumor-induced transfectants. No bands were present in DNA from NIH 3T3 controls. This suggests the presence of specific fish sequences in transformants, and analysis with specific oncogene probes suggests that those are not homologous to other known oncogenes.

We have also characterized the proto-oncogene ets-2 homolog isolated from a Xenopus laevis oocyte cDNA library. The open reading frame length of the frog ets-2 sequence is 472 amino acids. The putative initiation and termination codons are co-linear with the homologous human and mouse sequences. The entire cDNA sequence was cloned into a bacterial expression vector, and high-level expression of proto-oncogene-encoded protein was obtained. The ets-2-expressed protein is being purified to make antiserum and to study its biochemical properties. The expression pattern of the frog 3.2 kb ets-2 mRNA is typical of a maternal mRNA during oogenesis and embryonic development. The mRNA level (on an oocyte per embryo basis) remains almost constant during oogenesis; a similar level is maintained from the egg stage through early cleavage. The frog ets-2 mRNA was found to be nearly evenly distributed throughout the cytoplasm of the oocytes, and not specifically localized in the animal or vegetal pole. Injection of antisense oligonucleotides into oocytes

results in the degradation of the endogenous ets-2 mRNA and blocks germinal vesicle breakdown (GVBD) induced by hormone. Thus, the ets-2 product appears to be required for the meiotic maturation of Xenopus oocytes. We have expressed a full length copy of the Xenopus laevis ets-2 gene in bacteria. The expressed protein is recognized by a polyclonal antibody raised against an expressed segment of the Xenopus gene. A monoclonal antibody that recognized this protein, raised against a peptide from a region that is conserved among ets sequences, was identified. This antibody should be useful in the identification and characterization of the Xenopus ets gene product in oocytes. Having found that members of the ets family of oncogenes are present in Drosophila, the sea urchin and several vertebrates, we utilized a series of oligonucleotides based on a consensus of all ets sequences as probes to detect possible ets-related sequences in the yeast Saccharomyces cerevisiae. The identification of sequences that are homologous to different regions of these ets probes will make possible the molecular cloning and characterization of yeast proto-ets sequences.

Transfection of ets Proto-oncogenes

In an effort to study the role of the ets proto-oncogenes in cell proliferation and transformation, we have constructed vectors containing either the chicken ets-1 or mouse ets-2 proto-oncogene (c-ets) linked to the mouse metallothionein I promoter. NIH 3T3 cells transfected with either construct showed foci of densely-growing, morphologically-altered cells when cultured in low serum (0.05%) or serum-free medium. Control cells transfected with vector alone did not grow in serum-free medium, and the growth was slow in medium containing 0.05% serum. In order to test whether the growth alterations observed with c-ets-1- or c-ets-2-transfected cells are mediated by the expression of ets-1 and ets-2 genes, we have cloned and analyzed several foci for the presence and expression of metallothionein-linked ets genes. All foci examined contained multiple copies of the expected ets gene and expressed high levels of the appropriate ets-specific mRNA. Protein extracts prepared from ets-2-derived cell lines show a high level of the 56Kd ets-2 product. To test the ets-2 gene transfectants in these cells for transforming activity, we analyzed the growth of these lines in soft agar. Significantly, the ets-2- transfected cells formed colonies in semi-solid media and induced tumors in nude mice. A similar analysis for the ets-1-transfected cell lines is in progress.

Carcinogenesis is a multistep process and involves several independent steps. At the cellular level, it has been suggested that at least two oncogenes are required to convert a normal cell into a tumor cell. The oncogenes that complement each other in transformation of primary cells have been classified into two groups. The first group contains nuclear oncogenes, such as myc, p53, polyoma large T-antigen, jun, E1a of adenovirus, E7 of HPV; and the second contains cytoplasmic oncogenes, such as ras and middle T-antigen of polyoma. The c-ets-2 gene product ($p56^{ets}$) is localized in the nucleus, and it has been shown that the ets-2 gene is involved in cell proliferation. On the basis of nuclear localization and mitogenic activity, it has been suggested that ets-2 is a member of the nuclear oncogene family (p53, myc, fos, myb). Recently, we have performed an extensive search in the NBRF protein data base for secondary structure consisting of a negatively-charged

short turn, followed by an α-helix, and found that this motif is also present in nuclear oncoproteins, such as myc, ets-1, ets-2 and polyoma large T-antigen.

ets Oncoproteins

Having established that the ets gene family consists of a group of genes that are very highly homologous to the 3′ domain of the E26 viral oncogene, we generated a monoclonal antibody (MAb) against a synthetic oligopeptide identical to a hydrophilic and highly-conserved 3′ amino acid sequence located in the human ets-2 product. This MAb was shown to specifically react with all known ets proteins (p135 v-ets, ets-1, ets-2, erg-1, erg-2), as well as several other uncharacterized ets-related antigens. The ets-1 and ets-2 proteins were also identified using MAbs prepared against a bacterially-expressed ets-1 or ets-2 protein. The ets-1 monoclonal antibody identified a cytoplasmic p51 phosphoprotein and a nuclear p48 phosphoprotein, as well as a p42 and p39 non-phosphorylated nuclear proteins. The ets-2 MAb also identified a nuclear p54 protein. In cells expressing both ets-1 and ets-2, there is a ~10-fold higher amount of the ets-1 protein relative to the ets-2 protein. The ets-1 and ets-2 proteins were shown to have several properties in common with other known nuclear oncogenes, including low abundance, fast turnover, nuclear localization and response to mitogenic stimuli. Stimulation of the T-cell antigen receptor by antibody to the antigen receptor caused immediate Ca^{++}-dependent phosphorylation on serines of the p54 ets-2 and p51 and p48 ets-1. The p42 and p39 ets-1 proteins are not phosphorylated because they are lacking the putative phosphorylation site encoded by exon 7. Two-dimensional gel electrophoresis of these proteins manifests a negative charge with an apparent isoelectric point of less than pH 6.0. The phosphorylated forms of ets-1 and ets-2 have anomalous mobility on the two-dimensional gels, indicating that they are not fully denatured. Recently, we prepared a new monoclonal antibody specific to the proteins encoded by the ets-1 gene. This antibody recognizes multiple forms of the human ETS-1 protein (p52, p51, p49, p48, p41 and p39). Alternative splicing could be partly responsible for the generation of these multiple ETS-1 proteins because the p41 and p39 specifically lack the epitope encoded by exon 7 of human ETS-1 gene. Differential phosphorylation also contributes to the heterogeneity of the proteins; the p52 and p49 are the phosphorylated forms of p51 and p48, respectively. P41 and p39 are not phosphorylated. This phosphorylation is regulated by the mitogenic signals to T lymphocytes. When the human T cell, Jurkat, was treated by various mitogens, the ETS-1 proteins were quickly phosphorylated by a calcium-dependent mechanism. Experiments using another monoclonal antibody gave similar results with the proteins encoded by the ets-2 gene, an ets-1 related gene. The ets-2 protein is also heterogenous, consisting of two components, p56 and p54. Phosphorylation of the ets-2 proteins were stimulated by T cell mitogens in a calcium-dependent manner. These results suggest that the ets-1 and ets-2 proteins have a role in the calcium-mediated signal transduction in T lymphocytes. Their nuclear localization and DNA binding activity are consistent with the possibility that they take part in gene regulation linked to T cell activation. We have purified both ets-1 and ets-2 proteins by simple, rapid immunoaffinity chromatography using the monoclonal antibodies. These purified proteins will be useful in the functional characterization of the ets proteins. Thus, MAbs

to the ets-1 protein have demonstrated that the human ETS-1 gene product consists of multiple species of proteins, four major proteins and two minor proteins. By combination with the MAb and a set of antibodies against different epitopes of the ets-1 protein, two of four major proteins appeared to have arisen from mRNA devoid of exon 7, suggesting that these proteins were generated by alternative splicing. By subcellular fractionation and immuno-electron microscopy, the multiple ETS-1 proteins were found to be distributed both in the nucleus and cytoplasm. In vivo labeling with [^{32}P]-orthophosphate showed that the proteins were heterogeneously phosphorylated; two minor proteins were demonstrated to be phosphorylated forms of the upper 2 major proteins. The lower two major proteins, that appeared to have come from the deletion of exon 7, were not phosphorylated. Generation of multiple forms of ETS proteins and the heterogeneity of their localization and phosphorylation may imply some functional significance for these human ETS-1 gene products. Several approaches to investigate the cellular function of the ets gene products in T-cells and astrocytoma cells have been effected. Expression and phosphorylation of ets gene products in T-cells and astrocytoma cells during different stages of the cell cycle are being studied in CEM cells synchronized by centrifugal elutriation. Intracellular Ca^{++} will be measured fluoro-metrically in studies designed to elucidate the role of ets in Ca^{++}-mediated intracellular signaling pathways. The function(s) of the ets protein in astrocytoma cells are being analyzed using a variety of biochemical and cell biological techniques, including microinjection. All of these studies employ the antibodies and methods of ets protein isolation, previously developed in this laboratory. The murine ets gene products (ets-1 and ets-2) are expressed at higher levels in CD4+ CD8- thymocytes than in CD4+ CD8- T-cells. The effect of mitogens, lymphokines and activators of second messengers (activators of protein kinase C [PKC] and calcium-dependent protein kinase) was studied on isolated peripheral T-cells and in two different murine helper subset cell lines (Th1 and Th2) to dissect the role of ets gene products during T-cell activation and proliferation. The peripheral T-cells express more ets-1 than ets-2 mRNA. When quiescent T-cells are stimulated to proliferate with either cross-linking TCR/CD3 receptors or with a combination of PMA and ionomycin, the ets-2 mRNA is induced by 5- to 10-fold, whereas the ets-1 mRNA is reduced to basal levels. These results, in conjunction with the appearance of both ets-1 and ets-2 gene products during 3T3 fibroblast cell proliferation, further support the hypothesis that the ets-2 gene products play a role during the early phase of cell proliferation. Both murine ets-2 and human ERG cDNA clones have been expressed in prokaryotic expression systems in order to generate large amounts of proto-oncogene proteins. These polypeptides enabled us to prepare both monoclonal and polyclonal antisera. Characterization of monoclonal antibodies directed against various oligopeptides derived from the conserved domain of the ets family of genes is underway. Use of the ets and erg gene products and their specific antibodies as potential diagnostic markers for particular types of leukemias is under investigation.

c-myc Oncogene

A key role for the c-myc oncogene in cellular proliferation has long been postulated. C-myc may act by modulating the expression of other cellular genes whose products directly control proliferation. Permanent cell lines (in

which the endogenous c-myc gene is tightly regulated by growth factors and cell/cell contact) have been constructed in which expression of an exogenously transfected c-myc gene is controlled by the Drosophila heat shock 70 promoter. Transcription and subsequent translation of the exogenous c-myc gene is specifically induced by mild heat shock. The endogenous c-myc gene is not expressed under these conditions. When compared to heat-shocked cell lines which contain constructs lacking c-myc, several changes in cellular gene expression are observed: (1) two-dimensional analysis of the proteins from c-myc-containing cell lines shows the induction of eight protein species and the repression of five protein species relative to cell lines lacking c-myc; (2) the transcripts of two genes (3CH77 and 3CH92) previously identified as serum inducible are induced when c-myc is expressed; (3) the endogenous heat shock 70 gene may be specifically induced in response to c-myc. Therefore, c-myc expression alters the expression of other cellular genes, including the induction of some known to be expressed only in proliferating cells.

Oncogenes and Human Malignancy

The members of the human ETS gene family now account for five independent chromosomal loci: ETS-1 ETS-2, ERG, ELK-1, ELK-2, and have been localized at chromosome regions, some of which are of genetic interest, both for constitutional and acquired (neoplasia) genetic disease. We have focused on two of these regions, the 11q23 and 21q22 regions, where ETS-1 and ETS-2 have been mapped. The ETS-1 gene has been found (1) transposed, but not structurally altered in several translocations associated with acute non-lymphocytic leukemia, and transcribed at very low, but normal-sized levels in acute non-lymphocytic leukemias (ANLL), regardless of whether they are positive for translocations; (2) neither transposed nor structurally involved in Ewing's sarcoma, neuroepithelioma, Askin's tumor with (11;22) translocation and in the constitutional (11;22) chromosomopathy. The hypothesis of a relationship between ETS-2 and Down's syndrome (DS) has been tested on two grounds. While ETS-2 tested negative as a putative cis-acting genetic element, not interfering with correct chromosome 21 segregation at meiosis, it was shown to be a component of the minimal genetic region responsible for DS. Increased ETS-2 gene dosage, certainly extraneous to Alzheimer's disease (AD), is probably not the cause of AD developed by DS individuals. That higher ETS-2 gene dosage might be responsible for the post-natal predisposition to leukemia in DS remains to be proven. Nevertheless, we have shown that at least a second gene, different from ETS-2, of relevance to leukemogenesis, must reside on chromosome 21, since the t(8;21) breakpoint of acute myelogenous leukemia M2 is more than 17 cM proximal to ETS-2. Extension of this work--the introduction of the ETS-2 gene in transgenic mice--may serve as an animal model for testing the effect of additional ETS-2 gene products. Transgenic mice offer a powerful model system for studying the molecular mechanisms for gene regulation during development. To study the role of ets proto-oncogenes in the normal developmental processes and tumorigenesis, we have generated transgenic mice that contain ets-2 genes. The transgenic mice were generated by microinjection of ets-2 cDNA linked to the mouse metallothionein promoter into the pronuclei of one-cell embryos. The injected embryos were implanted into the oviduct of pseudopregnant mothers and brought to term. One of the 22 pups was shown to contain the transgene by Southern

blot analysis of DNA prepared from tails. The founder mouse (female) was bred again to produce offspring that contained the ets-2 transgene for further studies.

Colon cancer causes over 60,000 deaths in the United States each year and is second only to lung cancer as the cause of cancer deaths in the U.S. Patients with an inherited predisposition for developing hundreds of polyps (called familial polyposis) are at nearly 100% risk of colon cancer by age 40. A large number of colon cancers originate from mucosa (i.e., not from a polyp), especially in the proximal colon so that a prehistory of polyposis which might lead to early detection is lacking. Recently, restriction fragment length polymorphisms (RFLPs) linked to familial polyposis have been described. Elevated ras gene expression is also observed in a large fraction of polyps and carcinomas. A pattern of chromosomal abnormalities is beginning to emerge. In order to directly investigate the major alterations in gene expression in colon carcinoma and in polyps compared to normal colonic mucosa, cDNA libraries from matched tumor/normal tissues have been constructed. Tumor minus normal, and normal minus tumor subtractive cDNA libraries have been prepared and enriched for tumor-specific and normal-specific cDNAs, respectively. Two cDNA clones whose expression is greatly elevated in carcinoma tissue, but not in normal or polyp tissue, have been identified and isolated. Other clones from these differential cDNA libraries have been isolated and their expression levels are being characterized. Expression of these clones in colon carcinoma cell lines, as well as in other carcinomas (e.g., breast, ovary, endometrium) are being examined. One objective of the LMO is, therefore, to identify candidate tumor suppressor genes that may eventually yield markers to analyze human cancers. The strategy we used is to isolate morphologically-flat revertants from NIH 3T3 cells transformed by the EJ-ras oncogene, following transfection with a cDNA expression library containing tumor suppressor genes from normal cells. The functional cDNA clones can then be recovered from the flat revertant cells and further characterized. Using a methionine starvation procedure as a negative selection for flat revertants, we are able, in a typical experiment, to isolate more than 40 morphologically flat clones from EJ-ras-transformed cells following transfection with a cDNA library constructed from human fibroblasts. Experiments are now in progress to characterize these flat revertants and to recover the cDNA clones from the flat revertants in order to identify specific tumor supressor expressed product(s).

ras Oncogene

Another objective is to elucidate the molecular mechanism by which ras oncogenes transform cells into malignant phenotypes. One emphasis is directed toward delineating the structure-function relationship of the ras p21, and the signaling pathways in which ras participated to control cell growth and differentiation. By site-directed mutagenesis of ras oncogenes, the functional significance of the three consensus sequence elements (GXXXXGK, DXXG, NKXD) of the GTP-binding site of ras p21 has been investigated. Interestingly, some mutations in these elements result in dominantly-acting negative phenotypes that interfere with the normal function of ras genes. Presumably, some of these mutant proteins sequester vital cellular targets of ras function. Studies are in progress to use these mutants to identify p21

cellular targets. Since some mutants are lethal in yeast, and their products are potentially cytotoxic to recipient cells after transfection, a transient COS cell gene expression system is being used for these biochemical studies.

Point mutations have been created in the GTP-binding consensus regions of v-ras-H to study their effects on biochemical and biological properties of the ras-encoded protein, p21. Several of these mutant proteins do not bind GTP and, thus, lack GTPase activity, while others were shown to have their GTP binding reduced. We have introduced these ras mutants into NIH 3T3 cells to study parameters of transformation, particularly growth in low serum, metastasis, and tumorigenesis in mice. G418-resistant NIH 3T3 cell lines were derived by transfection with constructs obtained by inserting the mutant proviral DNA into the pSV2neo plasmid. Clones with valine mutation at positions 13 or 15 or tyrosine at position 116 were incapable of morphologically transforming cells, while all other mutants with GTP-binding activity were competent. Ras, with a valine mutation at glycine-10, which had lost its ability to bind GTP and its autokinase activity in vitro and in vivo, was fully capable of transforming NIH 3T3 cells.

Feline Immunodeficiency Virus (FIV)

Feline immunodeficiency virus (FIV) produces a pathological condition in cats similar to acquired immunodeficiency syndrome (AIDS) in humans, produced by human immunodeficiency virus (HIV). Therefore, FIV is a useful animal model for studying AIDS. The Crandell feline kidney (CrFK) tissue culture cell line can be productively infected with FIV. Virions from infected CrFK cells were isolated, the genomic RNA purified and a cDNA library constructed. A series of overlapping clones, representing at least 6Kbp of the 9Kbp genome, have been isolated. FIV cDNA clones hybridize only to RNA and DNA from infected CrFK cells, thus ruling out that endogenous retroviruses have been induced. Partial sequence analysis of some clones resulted in a sequence which does not correspond to any retroviral sequences present in GenBank. This result suggests that, like its human counterpart, HIV, the feline immunodeficiency virus may contain additional open reading frames relevant to its pathology.

Human Immunodeficiency Virus-1 (HIV-1)

Having pioneered in the development of prokaryotic expression vectors to produce large amounts of human retroviral-encoded gene products, we have utilized this system to express the HIV-1 env gene as nine polypeptide components spanning the entire gp120 and gp41 regions. These recombinant proteins were expressed at high levels; typically, ~2-15% of the total E. coli proteins could be produced as recombinant HIV-encoded env gene polypeptides. Some of the nine polypeptides were partially purified and then purified to homogeneity by using ion-exchange/gel-filtration chromatography or HPLC methodology. All of the polypeptides were characterized serologically by the Western blot transfer method using a panel of human HIV-1-positive test sera and normal sera as controls. With the exception of one polypeptide that contains the transmembrane portion of the gp41 env gene product, all of the expressed env products were immunoreactive using human sera from HIV-1-infected individuals in double-blind assays. Recently, we have produced

milligram quantities of several env gene-encoded polypeptides, particularly those located in the carboxyl-terminal region of gp120 that do not contain the CD4 homologous residues. Also expressed at high quantities and purified is an amino terminal polypeptide derived from gp41 that is very immunogenic and very highly reactive in double-blind screening assays with almost every sera tested thus far. A number of these expressed polypeptides were used as antigens to successfully generate polyclonal and monoclonal antibodies that have been immunologically characterized. To aid in the identification and mapping of important immunogenic epitopes for antibodies found naturally in human sera, we have begun the production of recombinant vector-expressed, envelope-encoded polypeptides that are C-terminal truncations of already existing HIV-1 env gene-encoded polypeptides. Using a truncated HIV-1 gp120-encoded polypeptide in Western blots enabled us to detect the presence of a shared epitope containing a common sequence (Asa, Asn, Lys, Thr) present in two other vector-expressed products; this epitope, we found, is also recognized by antibodies present in the sera derived from AIDS patients, as well as the serum obtained from Dr. D. Zagury (Universite Pierre et Marie Curie, Paris, France) who experimentally self-inoculated himself using a recombinant vaccine-expressed HIV-1 envelope protein. Dr. Zagury is presently testing a number of our expressed env gene products for immuno-reactivity using his own sera, as well as sera obtained in field trials in Western and Central Africa.

Human Immunodeficiency Virus-2 (HIV-2)

HIV-2, the West African counterpart of the AIDS virus, is related to, but quite distinct from HIV-1. The HIV-2 NIH-Z genome is approximately 9.4 kb long with an env gene of about 2.7 kb, which codes for an envelope protein of 856 amino acids. The gp160 envelope protein is matured into a 120 Kd exterior glycoprotein (gp120) and a transmembrane protein of 35 kd (gp35). The gp120 and gp35 envelope proteins of HIV-2 NIH-Z show about 32% and 39% homology, respectively, with the HTLV-IIIB strain of HIV-1. The chief objectives of this project are (1) to develop a diagnostic antigen for accurately detecting antibodies to HIV-2 in all infected human sera; and (2) to use the bacterially-expressed HIV-2 env gene products in some vaccine applications. We have expressed the env gene of the NIH-Z isolate of HIV-2 as five overlapping fragments in E. coli. The env open reading frames (ORFs) were initially expressed as tripartite fusions in between the 12 amino-terminal codons of the lambda cII gene and the lacZ (β-galactosidase) gene of E. coli, placed under the transcriptional control of the λ PL promoter on the expression plasmid pWS50. The β-galactosidase portion of the fusion protein was then deleted at the DNA level by generating in-frame translational stops or translational frame-shift mutations. One of the ORFs spanning the amino acid residues 536 through 705 of the HIV-2 env gene directed the synthesis of a 20 Kd protein that was serologically specific for identifying antibodies to HIV-2. This antigenic protein is produced at levels approximately 5% of the total cellular proteins. No significant immunological cross-reactivity was observed with HIV-1-positive sera or with normal control sera in immmunoblot assays with a crude preparation of this protein. Work is now in progress on the immunological characterization of the gene products of the remaining four HIV-2 env ORFs. The vector, pJL6, and its derivatives have also been used to express several nonstructural proteins from human immunodeficiency virus (HIV)

types 1 and 2 in order to assess any biological activities associated with these products. These proteins include the HIV-1 vpu and nef proteins, as well as the HIV-2 nef protein. The expressed proteins are of potential use as diagnostic reagents in HIV-infected individuals. We have found that 9 out of 29 HIV-1 sera (31%) reacted with the bacterially-expressed HIV-1 vpu proteins.

Molecular Biology of HIV

The expression of human immunodeficiency virus type 1 (HIV-1) in a variety of infected cells was studied by RNA gel blotting. The highest levels of expression were seen in HTLV-III-infected H9 cells and fresh peripheral blood lymphocytes (PBLs). Other strains of the virus were expressed in PBLs at a lower level. Virus-specific messages were detected in infected macrophages, but at a somewhat lower level than in PBLs, even when the virus analyzed was a macrophage-tropic strain. cDNA libraries have been constructed from infected and uninfected macrophages so as to allow the isolation of cellular sequences that are expressed at higher levels in infected cells. These sequences will be enriched by cDNA library subtraction and will be identified by differential hybridization.

In order to study the effect of the human immunodeficiency virus tat gene on the expression of cellular genes, we have constructed lymphoid lines that expressed the tat gene. This was done by placing the tat gene and an adjacent HIV LTR into the retroviral vector, pGV1. The recombinant plasmid was transfected into psi2 cells to produce an ecotropic viral stock that was used to infect psiAM cells. Colonies of G418-resistant psiAM cells were isolated and assayed for the production of virus. Supernatants of those colonies with the highest virus production were used to infect cells of the human T-cell lymphoid line, H9. G418-resistant cell lines derived from H9 were found to contain tat activity as assayed by fusion with HeLa cells containing LTR and CAT sequences. Furthermore, they contained an LTR-tat message of the predicted size.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

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Toward a Molecular Description of Malignant Transformation by p21 ras Oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
1.27	1.00	0.27

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The major objective of the present project is to elucidate the molecular mechanism by which ras oncogenes transform cells into malignant phenotypes. The present emphasis is directed toward delineating the structure-function relationship of the ras p21, and the signaling pathways in which ras participated to control cell growth and differentiation. By site-directed mutagenesis of ras oncogenes, the functional significance of the three consensus sequence elements (GXXXXGK, DXXG, NKXD) of the GTP-binding site of ras p21 has been investigated. Some mutations activate the oncogenic potential of the proto-oncogenes. Interestingly, some mutations in these elements result in dominantly-acting negative phenotypes which interfere with the normal function of ras genes. Presumably, some of these mutant proteins sequester vital cellular targets of ras function. Studies are in progress to use these mutants to identify p21 cellular targets. Since some mutants are lethal in yeast, and their products are potentially cytotoxic to recipient cells after transfection, a transient COS cell gene expression system is used for these biochemical studies.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

T. Y. Shih	Research Chemist	LMO NCI
L. Gutierrez	Visiting Fellow	LMO NCI
L. S. Ulsh	Microbiologist	LMO NCI
Y. Ogiso	Visiting Fellow	LMO NCI
A. Seth	Visiting Scientist	LMO NCI

Objectives:

The major objective of this project is to investigate the molecular biology of the ras oncogenes and their p21 products. The long-range goal is to elucidate molecular mechanisms of cell transformation induced by these gene products, with the purpose of contributing to our understanding of the roles of oncogenes in human carcinogenesis, and to develop strategies for human tumor detection, monitoring and intervention. Earlier work on this project has contributed to the identification of the ras oncogenes and their p21 products in Harvey and Kirsten murine sarcoma viruses (J Virol 1978;25:238-52; Virology 1979; 96:64-79), and characterization of the major properties of ras p21, i.e., activities associated with guanine nucleotide binding (Nature 1980;287:686-91). In collaboration with Drs. Papas, Lautenberger and Blair (see Projects Z01CP04899-17, Z01CP05120-10 and Z01CP05295-08), biochemically-active ras proteins overproduced in E. coli have been studied, and biological properties of ras oncogenes have been investigated. These studies have led to the current concepts that ras proteins function as cellular signal transducers, relaying extracellular growth control signals to intracellular effectors in control of cell proliferation. Many other studies have implicated the roles of ras oncogenes in various aspects of the multistep and multifactor process of human carcinogenesis (Ann Rev Biochem 1987;56:779-827). The major emphasis of the current study is directed toward elucidating the structure-function relationship of the ras proteins, and the molecular signaling pathways in which ras participated to control cell growth and differentiation.

Methods Employed:

1. Site-specific mutagenesis. The H-ras oncogene of the proviral pH-1 DNA was cloned into the single-stranded M13 phage. The specific amino acid of the p21 ras proteins was altered by oligonucleotide-directed mutagenesis of the M13 template DNA. The mutant ras was either reconstructed into the pH-1 clones for transfection assays or inserted into the pJL6 vector for overproduction of ras mutant proteins in E. coli.

2. Construction of mutant ras genes in mammalian expression vectors. For expression of mutant ras in NIH 3T3 cells, which could be subjected to inducible agents, two vectors were used: (1) pMAM is an expression vector containing the MMTV promoter and is inducible with dexamethasone; (2) pBMT2X is a vector

regulated by the metallothionein promoter, and is inducible with metal ions such as cadmium. Mutant ras genes were inserted into these vectors for transfection to NIH 3T3 cells. For transient expression of ras mutants, simian COS cells, which were transformed by a defective SV40, were used. Constructs of mutant ras genes with vectors containing the SV40 origin of DNA replication were used.

3. Transfection of NIH 3T3 cells. Transforming activities of mutant ras genes were evaluated by transfection into NIH 3T3, cells and transfectants were selected by the neo^R gene marker. Tumorigenicity was evaluated by growth in soft agar and induction of tumors in nude and Balb/c mice.

4. Transient expression in COS cells. Abundant mutant ras expression was achieved by transient expression in COS cells expressing SV40 T-antigen. Biochemical analyses of ras proteins were performed 48 hrs. after transfection.

5. Purification of p21 from E. coli. A recombinant p21 was overexpressed in E. coli carrying plasmid, pJLcIIras I, by raising the temperature from 31°C to 41°C. p21 was then purified to over 95% purity under non-denaturing conditions. Alternatively, p21 was isolated by cell lysis with lysozyme and NP40. After centrifugation, the protein was extracted from pellets with 8 M urea.

Major Findings:

p21 ras proteins contain three consensus sequence elements of the following amino acid residues: GXXXXGK, DXXG and NKXD, in their primary structures. These sequences are conserved among members of the superfamily of the GTP-binding proteins. Previous studies using site-directed mutagenesis of the viral ras oncogenes to alter specific amino acid residues of these consensus sequence elements have concluded that these residues are critical in the interaction of p21 with its GTP or GDP ligand--mutations of the conserved residues profoundly affect biological activities of ras proteins. In the three-dimensional structure of ras proteins determined by X-ray crystallography, these sequence elements interact with the guanine base and phosphoryl group of the GDP ligand. These interactions control the conformational changes of p21 molecules to perform the on/off switching function in the signaling mechanism that controls cell proliferation. Some mutations activate the oncogenic potential of the proto-oncogenes, and some mutations generated in our laboratory are inhibitory to ras transformation. Genetic studies of the related yeast YPT1 gene indicate that mutations of the NKXD sequence element result in a dominant lethal phenotype; and similarly, mutations of the yeast ras gene in the GXXXXGK element dominantly interfere with ras function in the signaling pathways. In the structurally related EF-Tu, NKXD mutation results in a tight non-productive complex with its nucleotide exchange factor, EF-Ts. Presumably, these dominant negative mutant proteins sequester their cellular targets from interaction with their normal GTP-binding proteins. Experiments are in progress using these mutants in a COS cell transient gene expression system to identify cellular target proteins for ras functions.

Another area of our studies concerns phosphorylation of ras proteins by protein kinase C, which is activated by tumor-promoting phorbol esters, and by protein kinase A stimulated by cAMP. Protein kinases phosphorylate ras proteins on serine residues at the structurally-significant, hypervariable regions of p21 close to the membrane attachment site at the C-terminus. To evaluate the biological effects of phosphorylation on ras functions, we have made mutations of the H-ras gene at the phosphorylation site, changing the serine residue into either alanine or cysteine. These mutant ras genes were constructed into the pMAM vector inducible with dexamethasone or into the pBMT2X vector inducible with cadmium ion. Cell lines expressing these genes were derived from NIH 3T3 cells following transfection with these constructs. Studies are in progress to evaluate the biological consequences of these mutations on ras function.

Publications:

Papas TS, Samuel KP, Lautenberger JA, Dubois GC, Zweig M, Showalter SD, Seth A, Fisher RJ, Fujiwara S, Schweinfest CW, Shih T, Ascione. Vector systems for the high-level expression of oncogenes, HTLV-I and HIV polypeptides. In: Albertini A, Paoletti R, Reisfeld RA, eds. Molecular probes: technical and medical applications. New York: Raven Press, 1989;79-90.

Saikumar P, Ulsh LS, Clanton DJ, Huang KP, Shih TY. Novel phosphorylation of c-ras p21 by protein kinases. Oncogene Res 1988;3:213-22.

Shih TY, Clanton DJ, Saikumar P, Ulsh LS, Hattori S. Structure and function of ras p21: studies by site-directed mutagenesis. In: Bosch B, Kraal B, Parmeggiani A, eds. Guanine-nucleotide binding proteins. NATO ASI SERIES. New York: Plenum Press (In Press)

Shih TY, Saikumar P, Clanton DJ, Ulsh LS. Novel phosphorylation of ras p21 and mutational studies. In: Spandidos D, ed. The ras oncogenes. NATO ASI SERIES. New York: Plenum Press (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05120-10 LMO

PERIOD COVERED: October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*: Expression of Retroviral and Oncogene Proteins in Bacteria

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. A. Lautenberger	Research Chemist	LMO	NCI
Others:	Z. Q. Chen	Visiting Associate	LMO	NCI
	L. Burdett	IRTA Fellow	LMO	NCI
	R. J. Fisher	Expert	LMO	NCI
	L. Virgilio	Biologist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*: Nucleic Acid Protein & Synthesis Laboratory, Program Resources, Inc. (G. C. DuBois, K. P. Samuel, S. D. Showalter, M. Zweig)

LAB/BRANCH: Laboratory of Molecular Oncology

SECTION: Carcinogenesis Regulation Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.99	0.99	1.00

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have expressed a full-length copy of the Xenopus laevis ets-2 gene in bacteria. The expressed protein is recognized by a polyclonal antibody raised against an expressed segment of the Xenopus gene. A monoclonal antibody raised against a peptide from a region that is conserved among ets sequences was identified that recognized this protein. This antibody should be useful in the identification and the characterization of the Xenopus ets gene product in oocytes.

We have used the expression system to express several retroviral proteins, including the tax gene of HTLV-1, the nef and vpu genes of HIV-1, and the nef gene of HIV-2. We have used polyclonal and monoclonal antibodies raised against these proteins to analyze the biochemical and biological properties of the authentic viral proteins. We found 9 out of 29 sera (31%) from HIV-1 infection. Preliminary characterization of the HIV-1 and HIV-2 nef products suggests that they bind GTP and GDP. The status of HTLV-I tax expression was studied during cell-free and cell-mediated infection of peripheral blood lymphocytes. Since some attempts to immortalize these cells by cocultivation with infected cells failed, in spite of demonstrable tax expression, production of this protein is not sufficient for immortalization.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. A. Lautenberger	Research Chemist	LMO NCI
Z. Q. Chen	Visiting Associate	LMO NCI
L. Burdett	IRTA Fellow	LMO NCI
R. J. Fisher	Expert	LMO NCI
L. Virgilio	Biologist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

The bacterially-synthesized proteins can be used for three types of applications. They are: (i) production of proteins for study of biochemical properties, (ii) production of selected antigens for detecting specific antibodies in sera, and (iii) as immunogens in raising antisera that can be used for the identification and characterization of "native" proteins.

Methods Employed:

1. Recombinant DNA procedures. The recombinant DNA procedures used were described by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982).

2. Purification of heterologous proteins expressed in bacteria. Plasmids containing DNAs inserted into the pJL6 vector (Lautenberger et al. Gene 1983;23:75-84) or one of its derivatives was introduced in Escherichia coli (E. coli) cells carrying a temperature-sensitive allele of the phage lambda repressor (cI857) on a defective prophage. Such bacteria were grown at 32° to an optical density at 590 nm of 0.5 and the culture temperature is then shifted to 42°. After shaking for 1 hr. at the elevated temperature, the cells were pelleted and proteins were extracted as described by Krippl et al. (Proc Natl Acad Sci USA 1984;81:6988-92. Generally, the expressed heterologous protein is found in the KSCN pellet and can be solubilized in 7 M urea or 7 M guanidinium thiocyanate.

3. Immunoblot detection of expressed heterologous proteins. Protein fractions were subjected to electrophoresis on 10% SDS-PAGE and immobilized on nitrocellulose filters. Strips containing the transferred proteins were reacted with diluted antibody reagents or human sera. Immune complexes were detected by incubation of the washed strips with 125-I Staphlococcus aureus protein A, followed by autoradiography.

Major Findings:

1. Expression of a full-length Xenopus laevis ets-2 protein in bacteria. Using a multistep cloning procedure, including the introduction of the correct amino-terminal sequences by use of synthetic oligonucleotides, a full-length copy of the Xenopus laevis ets-2 gene has been expressed in E. coli. The vector used was pJL6, which contains the bacteriophage lambda pL promoter.

The protein has the size predicted for the length of the expressed open reading frame, 56,000 Da. The protein was used to screen a panel of monoclonal antibodies (MAbs) raised against a peptide that is conserved among ets sequences (FKLSDPDEVARRW). Since the expressed protein should be similar antigenically to the authentic Xenopus ets protein, this antibody should be useful in the identification and characterization of the authentic protein.

2. Bacterial expression of the human immunodeficiency virus-1 (HIV-1) vpu protein. The open reading frame U (vpu) of HIV-1, with a potential for encoding a protein of 82 amino acids, was inserted into the bacterial-expression vector pJL6. The chimeric vpu protein was shown by protein gel immunoblots to react with antibodies in sera from HIV-1-infected individuals. Nine out of 29 sera (31%) from HIV-1 seropositive individuals (as determined by reactivity to gag or env antigens) had antibodies that reacted positively to the p10cII-vpu protein. None of the 10 sera from HIV-1 seronegative people reacted to this protein. These results suggest that the U open reading frame expresses an immunogenic protein during HIV-1 infection in vivo.

3. Bacterial expression of the HIV-1 and HIV-2 nef proteins. We have produced large quantities of a portion of the HIV-1 nef gene product by expression in E. coli using the vector, pJL6. The expressed protein contains 116 amino acids from nef starting at residue 92. The protein was purified to homogeneity by gel filtration and reverse phase HPLC. The purified protein was utilized to produce high-titer rabbit polyclonal antisera and several mouse MAbs that recognize nef in a strain-specific manner. The mouse MAbs developed against the bacterial nef have been used for the identification of the authentic nef proteins. We have evidence for four forms of nef, a p25 and p27 that each are resolved into two spots by isoelectric focusing on two-dimensional protein gels. The two sizes observed by SDS-PAGE may correspond to forms observed by Ahmad and Venkatsan (Science 1988;241:1481-5) who found that the smaller form began at position 20 of the larger form. The smaller form (p25) is the predominant form in lysates of cells that have been passaged with virus over a long period of time. The larger form is predominant in cells that have been freshly infected with virus that has not been extensively passaged in culture. We have used immunoaffinity columns containing one of the monoclonal antibodies to purify viral nef. We have preliminary evidence that the purified protein binds GTP and GDP. This is consistent with our report (Samuel et al., FEBS Lett 1987;218:81-6) that the nef gene has homology with genes for nucleotide binding proteins.

We have also expressed a complete copy of the HIV-2 nef protein in E. coli using our vector system. While this protein is synthesized in small quantities, we have purified microgram quantities by open column gel filtration chromatography and HPLC procedures. Like the HIV-1 nef protein, the HIV-2 nef protein has been observed to bind GTP and GDP in a filter binding assay. We are presently preparing larger quantities of the protein for determination of the binding constant. This will permit us to evaluate the significance of nucleotide binding by nef in the process of infection.

4. The effect of human T lymphotropic virus-I (HTLV-I) tax expression on viral infection and cellular immortalization. We have examined the role of the HTLV-I tax gene product (p40tax) in the early stages of T-lymphocyte

transformation. These studies made use of antibodies raised against a portion of the tax gene expressed in the vector, pJL6 (Samuel et al., Gene Anal Techn 1986;3:17-24). Human peripheral blood lymphocytes (with or without mitogen treatment) were exposed to virus in a cell-free manner by use of Transwell membranes. The virus was produced by the HTLV-I-infected cell line, MT-2, that was present in the upper chamber. We were able to demonstrate the presence of a stably-integrated viral genome by polymerase chain reaction (PCR) analysis. The cells exposed to virus expressed the cell surface markers, CD4, CD3, and Tac. Tac, which has been shown to be a 55 kD subunit of the IL-2 receptor, is an indicator of cell proliferation. However, no tax protein was detectable by immunofluorescence and the cells did not become immortalized. In contrast to the studies with cell-free transmission, we were able to immortalize fresh peripheral blood lymphocytes by cocultivation with infected cells in four out of seven attempts. In these experiments, we found expression of the HTLV-I tax protein at early times following cocultivation, whether or not the cells were immortalized. These studies suggest that tax is necessary, but not sufficient, for HTLV-I mediated transformation.

Publications:

Chen ZQ, Fujiwara S, Bhat NK, Lautenberger JA, Fisher RJ. Immunological detection of protein antigens after phenol-chloroform denaturation. Gene Anal Techn 1989 (In Press)

Lautenberger JA, DuBois GC, Samuel KP, Seth AK, Showalter SD, Schweinfest CW, Zweig M, Papas TS. Expression of human retroviral proteins in a heterologous system. In: Groopman JE, Chen I, Essex M, Weiss R, eds. Early human retroviruses: UCLA Symposium on Molecular and Cellular Biology, New Series, Volume 119, New York: Alan R Liss, Inc, 1989 (In Press)

Papas TS, Samuel KP, Lautenberger JA, DuBois GC, Zweig M, Showalter SD, Seth A, Fisher RJ, Fujiwara S, Schweinfest C, Shih TY, Ascione R. Vector system for the high-level expression of oncogenes, HTLV-I, and HIV polypeptides. In: Albertini A, Paoletti R, Reisfeld RA, eds. Molecular probes: technology and medical applications. New York: Raven Press, 1989;79-90.

Watson DK, McWilliams MJ, Lapis P, Lautenberger JA, Schweinfest C, Papas TS. Mammalian ets-1 and ets-2 genes encode highly conserved proteins. Proc Natl Acad Sci USA 1988;85:7862-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05238-08 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Transforming Genes of Acute Leukemia Viruses and Their Cellular Homologues

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. K. Watson	Research Microbiologist	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI
	A. Seth	Visiting Scientist	LMO	NCI
	G. Mavrothalassitis	Visiting Fellow	LMO	NCI
	C. L. Jorcyk	Biologist	LMO	NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (S. Showalter, F. Smyth)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.31	0.71	0.60

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The functional relationship between the onc genes of transforming retroviruses and their cellular prototypes has been facilitated by structural comparisons at the nucleic acid and predicted protein levels. We have determined the complete nucleotide sequence of the chicken mouse and human ets-1 and ets-2 genes and compared them to each other and to the ets gene of E26. The chicken ets-1 gene has v-ets homologous sequences in nine regions over 60 kb of genomic DNA. In addition, the cellular gene contains unique 5′ and 3′ sequences. These structural differences may be responsible for the oncogenic potential of this retrovirus. The human ETS-1 gene product is over 95% identical to the chicken ets-1 gene. The mammalian ets-2 genes from man and mouse encode for nearly identical amino acids and are over 90% conserved relative to the chicken ets-1 gene. The ets-2 gene appears to have mitogenic activity in transfected cells. Alignment of the predicted ets proteins suggests that three domains exist. The domain closest to the carboxyl-termini is highly conserved in all predicted gene products from species ranging from human to Drosophila. The domain located at the amino-terminal end of the ets proteins is more divergent, being highly conserved only between the same gene isolated from different species (e.g., chicken ets-1 vs. human ETS-1; mouse ets-2 vs. human ETS-2). The central domain encoding the ets proteins is found to be most divergent, even between ets family genes of the same species. Thus, ets represents a family of genes whose members are diverging at variable rates. Recombinant DNA technology will be used to generate mutants to evaluate the function of these three domains.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. K. Watson	Research Microbiologist	LMO NCI
T. S. Papas	Chief	LMO NCI
A. Seth	Visiting Scientist	LMO NCI
G. Mavrothalassitis	Visiting Fellow	LMO NCI
C. L. Jorcyk	Biologist	LMO NCI

Objectives:

The purpose of this investigation is to determine the relationship between v-ets and its cellular homologues. Structural analysis of these genes will allow us to better understand their biological functions. In addition, we plan to evaluate the involvement of proto-ets genes in human malignancy.

Methods Employed:

1. Preparation of high molecular weight DNA from eukaryotic cells by treatment with proteinase K, followed by phenol-chloroform extraction. Vector DNA was prepared from phage and plasmid derivatives by phenol extraction and CsCl gradient centrifugation.

2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments on agarose and/or polyacrylamide gels. Specific DNA fragments were purified by electroelution or by extraction of low melting agarose.

3. Preparation of DNA probes using purified onc-specific DNA by nick-translation using E. coli DNA polymerase and DNaseI.

4. Preparation of nitrocellulose filters containing immobilized restriction fragments by the Southern blot technique and hybridization of onc probes.

5. Construction of recombinant phage libraries by ligation of restriction fragments of eukaryotic DNA to cosmid or λ vector DNA followed by production of phage by in vitro packaging.

6. Isolation of phage from the libraries containing virus-related sequences by hybridization of onc-specific probes to nitrocellulose filters containing phage DNA prepared from plaques lifted from petri plates by the procedure of Benton and Davis (Science 1977;196:180-2). Isolation of plasmid DNA from colonies lifted from plates by the method of Grunstein and Hogness (Proc Natl Acad Sci USA 1975;72:3961-5).

7. Subcloning of isolated DNA fragments into appropriate plasmid vectors, as required.

8. DNA sequence analysis of cloned DNA by the method of Maxam and Gilbert (Methods Enzymol 1980;65:499-560) and/or Sanger (Proc Natl Acad Sci USA 1977; 75:5463-7). In addition, uniquely labeled DNA will be sequenced following RNA-directed primer extension.

9. Total cellular RNA from cultured cells or tissues was prepared by the guanidine isothiocyanate method. In addition, RNA was prepared after cellular fractionation in the presence of RNase inhibitors. Separation of polyA+ and polyA- RNA by two cycles of purification through oligo(dt) cellulose. Characterization of RNA by electrophoresis on formaldehyde-agarose gels and Northern analysis.

10. Construction of cDNA library: Double-stranded cDNA was prepared from polyA+ RNA and ligated into λgt10 vector DNA for amplification.

11. Cloning of onc-genes and proto-oncogenes into prokaryotic vectors capable of overexpression of inserted DNA. Isolation of proteins used for antigens and for functional studies.

12. Controlled expression of oncogenes in eukaryotic vectors, using constructions with regulatable promoters. Transfection of eukaryotic vectors in mammalian cell lines for identification of expressed product(s) and for analysis of possible biological activity associated with the construct.

13. Identification for promoter and regulatory sequences present in genomic clones of the cellular homologs of oncogenes. To include nucleotide sequence analysis and functional assays using expression of defined reported genes, such as chloramphenicol acetyltransferase (CAT).

14. Nuclease protection assays to define the 5′ and 3′ ends of transcripts. Verification by sequence analysis of cDNA clones and by primer-extension of mRNA.

15. Polymerase chain reaction (PCR) to analyze tissue-specific splicing of the ets genes.

Major Findings:

1. Sequences related to ets (one of the two cellular genes present in the avian retrovirus, E26) have been detected by restriction enzyme digestion and Southern blot analysis using genomic DNA derived from vertebrates (avian and mammalian species) and invertebrates (Drosophila).

2. Chicken, mouse and human genomic libraries were screened and specific clones have been isolated. In addition, human, chicken and mouse cDNA clones have been isolated and subjected to nucleotide sequence analysis. Additional ets clones are being isolated and characterized for complete understanding of the molecular structure of the RNA products of the ets genes.

3. Chicken DNA segments homologous to the ets region were molecularly cloned and shown to be almost identical to v-ets by sequence analysis. The chicken ets locus from which E26 transduced ets sequences is designated chicken ets-1, and this locus is over 60 kb. Alignment with v-ets demonstrates that the viral homologous sequences are found in nine regions, each region bordered by consensus splice signals.

4. The chicken and viral ets genes are not homologous at the 3′ end; thus, the transforming protein of E26, p135 and the cellular ets gene product have different carboxyl termini.

5. Northern analysis of chicken thymus RNA, using probes from the first two viral homologous regions, failed to detect the major chicken c-ets transcript (7.5 kb). These regions are also absent in RNA prepared from chicken brain, heart, liver, gizzard and bursa. They are present in the RNA prepared from spleen, and represent an alternately-spliced species. Alignment of the sequence of this tissue-specific transcript with that of the genomic clones illustrates that an additional exon is present in the DNA. Using synthetic oligonucleotides identical to this unique 5′ exon, the localization of this novel exon has been determined and verified by sequence analysis. This exon is found over 5 kb upstream from homologous region I.

6. Chicken thymus RNA was used to prepare a complementary DNA library for isolation of an ets cDNA clone. Sequence analysis of an ets cDNA defines the complete open reading frame as including 441 amino acids. With the exception of 27 amino and 13 carboxyl terminal amino acids, the chicken proto-ets gene is nearly identical to the v-ets, containing only three nucleotide differences. The exon encoding the 27 unique amino acids is located between viral homologous regions II and III, about 20 kb upstream from region III.

7. cDNA clones for human and mouse ets-1 and ets-2 loci have been isolated and sequenced. This analysis demonstrated a strong conservation of amino acids (over 90%), suggesting that these genes perform an important function. The predicted amino acids encoded by the 469 (human) and 468 (mouse) ets-2 genes are similar (91% identity).

8. The predicted ets-2 proteins possess three distinct domains when compared to v-ets. The domain closest to the carboxyl-termini is highly conserved (>90%) and this conservation is seen to be widely preserved throughout evolution, including Drosophila. The domain located at the amino-terminal end of ets-2 is less homologous to the virus/chicken proto-oncogene and, thus far, this region has not been identified in lower eukaryotes. The third domain, which is located centrally, is diverged in ets-2 genes, but is conserved in the ets-1 gene.

9. The predicted human ETS-1 protein is nearly identical to the 441 amino acids of the chicken ets-1 gene, having over 95% amino acid in common.

10. The complete cDNA clones from human ETS-1 and ETS-2 have been used as probes to isolate the respective genomic clones from these loci. These clones will serve to allow the (1) determination of the precise molecular structure of the genes, i.e., the distribution and size of the exon of these loci; (2) determination of the mechanism of RNA generation by the genes, i.e., transcriptional start site(s) and polyadenylation sites; and (3) identification and molecular dissection of the promoter region of both of these genes. From this analysis of the human ETS-2 gene, the origin of three unique transcripts can be attributed to differential use of unique polyadenylation signals. These signals have been identified by both S1 nuclease protection and sequence analysis.

11. Both genetic loci are transcriptionally active in birds and mammals, yielding distinct products. The expression of ets-1 and ets-2 genes in human cell lines has been assayed by Northern blot analyses. The pattern seen suggests that these two genes are independently regulated.

12. Viral ets fragments, and human ets exons and cDNA have been placed in appropriate systems for protein overexpression and these proteins have been utilized to elicit antibody response.

13. The entire coding segment of the mouse and human ets-2 genes has been inserted into a eukaryotic expression vector under the transcriptional control of the metallothionein promoter. Such constructs have been microinjected into fertilized mouse eggs to assess the role of ets-2 during development and to establish cell lines with inducible ets-2. Also, transfection of NIH cells with this construct yields foci when cells are grown in serum-free media.

Publications:

Papas TS, Blair DG, Fisher RJ, Watson DK, Sacchi N, Fujiwara S, Bhat N, Ascione R. The ets genes. In: Reddy EP, Skalka A, Curren T, eds. The oncogene handbook. New York: Elsevier, 1988;467-85.

Seth A, Watson DK, Blair DG, Papas TS. C-ets-2 proto-oncogene has mitogenic and oncogenic activity. Proc Natl Acad Sci USA (In Press)

Seth A, Watson DK, Blair DG, Thompson D, Dunn KJ, Papas TS. C-ets-2, a member of Down's syndrome locus has mitogenic and oncogenic activity. In: Rotundo RL, Ahmad F, Bialy H, Black S, Brew K, Chaitin MH, Glaser L, Magleby KL, Neary JT, Van Brunt J, Whelan WJ, eds. Advances in gene technology: molecular neurobiology and neuropharmacology. Oxford/Washington DC: IRL Press, 1989;155.

Seth A, Watson DK, Fujiwara S, Showalter S, Papas TS. Expression of human and viral ets genes in E. coli; production of human ets-2 specific monoclonal antibodies. Oncogene Res (In Press)

Watson DK, Ascione R, Papas TS. Molecular analysis of the ets genes and their products. In: Pimentel E, Perucho M, eds. CRC critical reviews in oncogenesis. Miami: CRC Press (In Press)

Watson DK, McWilliams MJ, Lapis P, Lautenberger JA, Schweinfest CW, Papas TS. Mammalian ets-1 and ets-2 genes encode highly conserved proteins. Proc Natl Acad Sci USA 1988;85:7862-6.

Watson DK, McWilliams MJ, Papas TS. A unique amino terminal sequence predicted for the chicken proto-ets protein. Virology 1988;167:1-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

ZP1CP05295-08 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Studies on the Activation of Oncogenes in Viruses and Human Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. G. Blair	Supv. Research Chemist	LMO	NCI
Others:	K. J. Dunn	Bio. Lab. Tech. (Micro)	LMO	NCI
	Y. Lu	Visiting Fellow	LMO	NCI
	D. J. Clanton	Senior Staff Fellow	LMO	NCI
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COOPERATING UNITS (if any) Mol. Mech. of Car. Lab., Basic Res. Prog., BRI, Frederick, MD (G. Vande Woude, R. Paules, M. K. Oskarsson); Nucl. Acid & Prot. Syn. Lab (M. Zweig, S. D. Showalter, D. O. Halverson, L. A. Eader), Recomb. DNA Lab. (T. Wood), Lab. Cell. Molec. Struct. (M. Dean, W. Modi), PRI, Frederick, MD

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.45	2.00	0.45

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

We have continued to analyze the DNA sequences associated with the ovc oncogene, a human transforming sequence activated during DNA transfection and derived from the human ovarian carcinoma cell line, OVCAR-3. We have mapped a portion of that gene to band 9p24 by in situ analysis, and have identified a restriction fragment length polymorphism using this probe which will be a useful genetic marker in segregation studies involving human chromosome 9. Sequence analysis of the entire 2.2kb fragment from chromosome 9 reveals no significant homology to known genetic sequences.

We have detected a transfectable sequence in a spontaneously tumorigenic human cell line which induces tumorigenicity and serum-independent growth, but not morphological transformation, in NIH 3T3 mouse fibroblasts. Hybridization analysis with oncogene and growth factor probes suggests this represents a potentially new oncogene or growth factor sequence.

We have demonstrated that microcell transfer can be used to detect oncogene sequences present in human cell lines. We have demonstrated that this method results in the transfer of larger unrearranged segments associated with the selected oncogene than either chromosome transfer or DNA transfection.

We have constructed a hygromycin selectable retroviral vector which expresses activated human rasH, which can be used in conjunction with other selectable retroviral vectors, to study the effects of multiple oncogene expression in infected cells. We have shown that low levels of activated p21ras will not induce the tumorigenic phenotype in murine and human cells, suggesting a threshold level of even activated ras is required to transform susceptible cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. G. Blair	Supv. Research Chemist	LMO	NCI
K. J. Dunn	Bio. Lab. Tech. (Micro)	LMO	NCI
Y. Lu	Visiting Fellow	LMO	NCI
D. J. Clanton	Senior Staff Fellow	LMO	NCI
E. Priel	Visiting Scientist	LMO	NCI

Objectives:

To understand the mechanism of transformation by murine sarcoma viruses (MSV) and the function of specific viral and cellular gene products in this process.

To develop screening and selection systems to identify and isolate human transforming DNA sequences from primary human tumors and tumor cell line DNAs. To identify, isolate and characterize such sequences and their gene products. To characterize the normal cellular homologs of such sequences and to determine the mechanism by which their oncogenic potential is activated.

Methods Employed:

Standard methods of tissue culture of transformed and normal cells, DNA and RNA isolation from tumor tissues and tissue culture, Southern and Northern blotting using radioactive probes prepared from cloned DNAs, calcium-phosphate-DNA transfection, microcell and somatic cell fusion mediated gene transfer immunoprecipitation and protein gel analysis to detect the expression of specific cellular proteins.

Major Findings:

1. The ovc 2.2kb fragment has been localized by in situ hybridization to 9p24 and detects an RsaI restriction fragment length polymorphism. We had previously identified an oncogenic sequence, designated ovc, by DNA transfection of the human ovarian carcinoma cell line, OVCAR-3, and shown that a cloned 2.2kb fragment of this sequence is expressed in the parental OVCAR-3 cell line, as well as several other human tumor cells in culture. Hybridization to panels of human-mouse and human-hamster hybrids had localized this fragment to human chromosome 9. In situ hybridization has now further localized the sequence to band 9p24. This region has not previously been associated with known oncogenes, although deletions of 9p21-9pter have been described in some acute lymphocytic leukemias and acute non-lymphocytic leukemias. A restriction fragment length polymorphism (RFLP) was also detected with the ovc 2.2kb fragment probe following digestion with the enzyme, RsaI. In analysis of 68 chromosomes from unrelated individuals, the RsaI alleles were detected with frequencies of 0.35/0.65 and were heterozygous in 41% of the individuals tested. The entire 2.2kb fragment of the ovc oncogene

has been sequenced and the sequence compared to known gene sequences in GenBank. No significant homology was detected, confirming previous hybridization and chromosome localization data which indicated that this gene has not been previously identified or characterized.

2. A spontaneously tumorigenic derivative of the non-tumorigenic human tumor line, HOS, contains a transferable oncogenic activity which induces both tumorigenicity and the capacity for serum-free growth when transfected into NIH 3T3 cells. In the course of tumorigenicity studies using the human osteosarcoma cell line, HOS, we isolated a spontaneously-transformed variant which had acquired the ability to induce tumors in nude mice with a 6- to 10-week latency. The cells were identical to normal HOS cells by morphology, the presence of specific marker chromosomes, and by DNA fingerprint analysis using probes which detected hypervariable repeat sequences. However, tumorigenic HOS cells could reproducibly transfer the tumorigenic phenotype to NIH 3T3 cells through multiple rounds of DNA transfection. The tumorigenic mouse cells were morphologically normal, but had acquired the ability to grow in a defined media (QBSF-51, Quality Biological, Inc.) in the absence of protein growth factors, and media from these cells stimulated normal NIH 3T3 cells to grow in the absence of serum. Southern analysis using a panel of DNA probes specific for known oncogenes and growth factors (ras, met, sis, myc, raf, fgfIII, hst) has failed to detect evidence that the transfected gene is related to any of these known oncogenic sequences.

3. Microcell-mediated transfer of micronuclei prepared from the transformed human tumor cell line, MNNG-HOS, demonstrates that the transformed phenotype can be transferred using this technique and that more than 1Mb of linked genetic material can be identified and mapped. We have optimized techniques of micronucleation, microcell purification, and microcell transfer in order to evaluate the usefulness of this technique in the detection and identification of large oncogenes in transformed human and animal cells. In order to mark individual human chromosomes with a drug selectable marker, MNNG-HOS cells were infected with a retrovirus carrying the neo gene, which induces resistance to the drug, G418. G418-resistant MNNG-HOS cells were then used to generate microcells, and these microcells were fused to NIH 3T3 cells. The resulting hybrids were either selected for their transformed morphology or drug resistance, and the selected hybrids were characterized. Stable hybrids containing single human chromosomes could be isolated which are resistant to G418. Morphologically-transformed hybrids were also identified which contained sequences homologous to the activated met oncogene. Analysis with a series of probes to sequences linked to the met oncogene showed that cells from individual foci contained different amounts of apparently unrearranged DNA linked to the oncogene and that larger segments were present in microcell transformed cells than in those generated by either DNA or chromosome-mediated transfer. Data from these hybrids predicted a gene order consistent with other studies of the activated met oncogene, but not in agreement with some studies of the normal met locus.

4. Murine and human cells can be generated which express low levels of the activated human H-ras gene, but are neither morphologically transformed nor tumorigenic in nude mice, while cells expressing high levels of the gene express a fully-transformed phenotype. We have constructed a retroviral vector which induces resistance to the drug, hygromycin, and expresses the human ras^H gene derived from the T24 human bladder carcinoma. Both NIH 3T3 and HOS (human osteosarcoma) cells transfected with this construct express a tumorigenic phenotype and the NIH 3T3 cells are morphologically transformed. In contrast, cells infected with the defective retrovirus generated by this construct are neither tumorigenic nor morphologically transformed. Expression of Ha-$p21^{RAS}$ in infected cells was confirmed by immunoblot analysis, but the level of expression was 5- to 10-fold lower than that observed in transfected cells. This data is consistent with data from studies of ras transformation in several systems, which suggests that the level of even activated ras expression is critical for the induction of the transformed phenotype.

5. Restriction of the feline endogenous virus RD114 in cat cells does not involve inhibition of RD114 LTR-mediated RNA expression. The analysis of how natural cellular mechanisms control viral infection and expression may provide valuable insights into new approaches to control pathogenic retroviruses. Previous evidence had suggested that the inability of the feline endogenous virus, RD114, to replicate in cat cells did not involve restriction at the level of virus uptake (Fischinger et al., J Gen Virol 1975;29:51-62). The availability of a permissive cat cell variant (G355) and recombinant clones of both the non-restricted feline leukemia virus (FeLV) and RD114 allowed us to investigate the molecular nature of RD114 restriction. We have confirmed earlier data that feline cells possess a receptor for the RD114 envelope gp70 and can be efficiently infected by viral pseudotypes packaged by RD114. We have also shown that feline embryo fibroblasts (FeF), which cannot be efficiently infected by the RD114 virus, will produce high levels of RD114 if the proviral genome is introduced by transfection. Consistent with this observation, we have shown that chloramphenicol acetyl transferase (CAT) under the control of RD114 LTR seqences is expressed as efficiently in non-permissive FeF cells as is CAT under the control of a non-restricted FeLV LTR. These results are consistent with the hypothesis that RD114 infection of non-permissive cells is restricted at an early post-penetration stage, perhaps at the point of proviral integration.

Publications:

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Ihle JN, Smith-While B, Sisson B, Parker D, Blair DG, Shultz A, Kozak C, Lansford RD, Askew D, Weinstein Y, Isfort R. Activation of the c-H-ras proto-oncogene by retroviral insertion and chromosomal rearrangement in a MoLV-induced T-cell leukemia. J Virol (In Press)

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Papas TS, Blair DG, Fisher RJ, Watson DK, Sacchi N, Fujiwara S, Bhat NK, Ascione R. The ets genes. In: Reddy EP, Skalka A, Curran T, eds. The oncogene handbook. New York: Elsevier, 1988;467-555.

Paules RS, Propst F, Dunn KJ, Blair DG, Kaul K, Palmer AE, Vande Woude GF. Primate c-mos proto-oncogene structure and expression: transcription initiation both upstream and within the gene in a tissue-specific manner. Oncogene 1988;3:59-68.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05440-05 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Site-Directed Mutagenesis of ras Oncogenes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. J. Clanton	Senior Staff Fellow	LMO	NCI
Others:	D. G. Blair	Supv. Research Chemist	LMO	NCI
	T. Y. Shih	Research Chemist	LMO	NCI
	L. S. Ulsh	Microbiologist	LMO	NCI
	Y. Lu	Visiting Fellow	LMO	NCI

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1.33	1.00	0.33

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Point mutations have been created in plasmids containing v-ras-H to study their effects on biochemical and biological properties of the ras-encoded protein, p21. Several of these mutant proteins do not bind GTP and, thus, lack GTPase activity, while others were shown to have their GTP binding activity reduced. We have introduced these ras mutants into NIH 3T3 cells to study parameters of transformation, particularly growth in low serum, metastasis, and tumorigenesis in mice.

G418-resistant NIH 3T3 cell lines were derived by transfection with constructs obtained by inserting the mutant proviral DNA into the pSV2neo plasmid. Clones with valine mutation at positions 13 or 15 were incapable of morphologically transforming cells, while all other mutants with GTP-binding activity were competent. Ras, with a valine mutation at glycine-10, which had lost its ability to bind GTP and its autokinase activity in vitro and in vivo, was fully capable of transforming NIH 3T3 cells.

We have found no direct correlation between the ability of p21 to bind GTP and the mutant cell's ability for tumorigenesis and metastasis. Tumorigenic properties are lost in stages unrelated to GTP binding or gross morphological transformation. Not all morphologically-transformed cells were capable of metastasis.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. J. Clanton	Senior Staff Fellow	LMO NCI
D. G. Blair	Supv. Research Chemist	LMO NCI
T. Y. Shih	Research Chemist	LMO NCI
L. S. Ulsh	Microbiologist	LMO NCI
Y. Lu	Visiting Fellow	LMO NCI

Objectives:

The objective of this project has been to study the biochemical basis of oncogene activation of *ras* genes, as well as the biological consequences of a series of laboratory-controlled mutations that have altered the biochemical properties of viral Harvey *ras* (v-ras^H). Point mutations can be designed to affect the binding of GTP to the *ras* gene product, p21 (a process that appears to be important in the normal function of p21). These mutations can be introduced into NIH 3T3 cells and used to study various parameters of morphological transformation, such as tumorigenicity in athymic and immunocompetent mice, growth in low serum, and metastasis.

Methods Employed:

Recombinant DNA technology was used to construct plasmids containing the *ras* gene and pSV2*neo* (for drug resistance to the antibiotic, G418). Standard cell transfections (J Virol 1980;35:76-92) were performed to transfect plasmids into NIH 3T3 cells. Athymic and Balb/c mice were inoculated subcutaneously for tumorigenic assays. Mice were also inoculated via tail vein for metastatic assays.

Other methods used were gel electrophoresis, Southern blot hybridization and standard immunoprecipitation (Proc Natl Acad Sci 1986;83:5076-80).

Major Findings:

1). We have found that there is no correlation between the ability of ras mutations to bind GTP and transformation. In most cases, GTP binding is necessary for p21 function, and mutations of *ras* which destroy GTP binding are incapable of morphological transformation. Our analysis has revealed that three mutants (13V, 15V and 116Y) are incapable of binding GTP and so do not morphologically transform cells. The 10V, however, is fully capable of morphological transformation, but has no affinity for GTP, while mutations 117Q, 33H and 22K still bind GTP at a reduced rate and induce morphological transformation. We have demonstrated that all are capable of tumor formation in nude mice, although at different rates. Since several of these cell lines (13V, 15V and 116Y) are not morphologically transformed and form tumors slowly, we attempted to rule out the possibility that tumorigenesis was due to independent spontaneous changes in the populations of cells transfected by these three mutants, and not by the presence of the v-*ras* gene.

Our ability to use genomic DNA from 13V tumors to generate a second cycle of ras containing tumorigenic cells appears to support the hypothesis that the v-ras^H gene carrying the 13V mutation is responsible for the tumorigenic phenotype of 13V cells. However, we were unable to generate tumorigenic transfectants using 116Y genomic DNA, and 15V cells induced several tumors which appeared after long latent periods and which did not contain transfected sequences. Thus, it is possible that the p21 produced by either of these mutants is not capable of inducing any detectable transformed phenotype.

2). The properties of the mutant v-ras transformed cells we have described suggest that the ability of the activated v-ras gene to induce the various characteristics of the transformed phenotype (metastasis, tumorigenicity, morphological transformation, serum independence) is lost in stages. The most active transforming genes are those that are capable of metastasis and growth of tumors in immunocompetent mice. These include the unmodified v-ras (N/R), and three which carry additional modifications (10V, 515 and 33H). Less potent ras mutants form tumors only in nude mice (117Q, 22K, 13V and 116Y). Of this group, several retain the ability to transform cells (117Q and 22K). Weakly transforming mutants (13V and 116Y) only retain the ability to produce tumors in nude mice. The 15V was incapable of forming tumors that retained any v-ras sequences. Finally, only 13V of this group can stably maintain its phenotype through a cycle of serial transfection.

3). Weakly transforming mutants block the ability of unmodified v-ras^H to induce morphological transformation when the two plasmids are co-transfected into NIH 3T3 cells. When 200 ng of N/R plasmid is co-transfected with 2 μg of 116Y plasmid, there was complete and reproducible suppression of foci. Two-hundred ng of N/R plasmid without 116Y produce 191 foci. Control co-transfection using pSV2neo showed no reduction in focus formation, indicating that the interference was not the non-specific result of the presence of a second neo-containing plasmid. It is possible that the 116Y p21 protein competes with the v-ras protein for some target proteins involved in the transformation process. The 13V mutation inhibits v-ras^H by 78%, and 15V mutants inhibit focus formation 95%. The recent finding that proteins similar to the ras protein can suppress the transformed phenotype lends credence to the hypothesis that 116Y might act in a similar fashion. The 116Y protein may interfere with some, as yet, unknown target in the pathway to transformation, or it may compete for a protein-like GAP which appears to be necessary for transformation.

Publications:

Saikumar P, Ulsh L, Clanton DJ, Huang K-P, Shih TY. Novel phosphorylation of c-ras p21 by protein kinases. Oncogene Res 1988;3:213-22.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05441-05 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of the Gene Products of the c-myc Locus and the c-ets Locus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. J. Fisher	Expert	LMO	NCI
Others:	S. Fugiwara	Visiting Associate	LMO	NCI
	T. S. Papas	Chief	LMO	NCI
	A. Seth	Visiting Scientist	LMO	NCI
	L. Fleischman	IRTA Fellow	LMO	NCI
	S. Koizumi	Visiting Fellow	LMO	NCI

COOPERATING UNITS *(if any)*
Nucleic Acid and Protein Synthesis Laboratory, Program Resources, Inc., Frederick, MD (M. Zweig, G. Dubois, N. K. Bhat and S. Showalter), Centro de la Investigacions, Madrid, Spain (S. M. Diaz de la Espina)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Transfenic Analysis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.86	0.86	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ets gene family consists of a group of genes which are highly homologous to the 3' domain of the oncogene carried by the avian acute leukemia virus, E26. A monoclonal antibody corresponding to a peptide in the hydrophilic and highly conserved 3' amino acid sequence of the human ets-2 was shown to specifically react with all know ets proteins (p135 v-ets, ets-1, ets-2, erg-1, erg-2), as well as several other uncharacterized ets-related antigens. The ets-1 and ets-2 proteins were identified by use of monoclonal antibodies prepared against a bacterially-expressed ets-1 or ets-2 protein. The ets-1 monoclonal antibody identified a cytoplasmic p51 phosphoprotein and a nuclear p48 phosphoprotein, as well as p42 and p39 non-phosphorylated nuclear proteins. The ets-2 monoclonal antibody identified a nuclear p54 protein. In cells expressing both ets-1 and ets-2 there is about a 10-fold higher amount of the ets-1 protein relative to the ets-2 protein. The ets-1 and ets-2 proteins were shown to have several properties in common with other known nuclear oncogenes, including low abundance, fast turnover, nuclear localization and response to mitogenic stimuli. Stimulation of the T-cell antigen receptor by antibody to the antigen receptor caused immediate Ca++-dependent phosphorylation on serines of the p54 ets-2, and p51 and p48 ets-1. The p42 and p39 ets-1 proteins are not phosphorylated because they are lacking the putative phosphorylation site in exon 7. Two-dimensional gel electrophoresis of these proteins show their negative charge with an apparent isoelectric point of less than pH 6.0. The phosphorylated forms of ets-1 and ets-2 have anomalous mobility on the two-dimensional gels, indicating that they are not fully denatured.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. J. Fisher	Expert	LMO	NCI
S. Fujiwara	Visiting Associate	LMO	NCI
T. S. Papas	Chief	LMO	NCI
A. Seth	Visiting Scientist	LMO	NCI
L. Fleischman	ITRA Fellow	LMO	NCI
S. Koizumi	Visiting Fellow	LMO	NCI

Objectives:

To characterize the protein products of the human proto-oncogenes, c-myc, c-ets-1 and c-ets-2. The characterization includes identification, subcellular localization, and determination of function for the normal gene products.

Methods Employed:

Biological Materials: Cell lines are used for enriched sources for the ets and ets-related proteins, including the human adenocarcinoma line, COLO 320, the human T-cell leukemia line, CEM, and the Burkitt's lymphoma line, Daudi. The COLO 320 cells are a rich source of myc proteins, ets-2 and erg proteins. Daudi cells are a source of ets-1, and CEM cells produce both ets-1 and ets-2. Normal tissues, such as mouse thymus or calf thymus, are used as an enriched source of c-ets proteins.

Protein Isolation: We have found three methods to extract the ets-2 proteins from cells. The cells or tissues are subfractionated into nuclear and cytoplasmic fractions. The nuclei (containing the majority of the ets-2) are extracted with urea, low ionic strength buffers or with 0.42 M NaCl. Our initial studies used urea-denatured extracts which allowed the isolation of an ets-related protein using fast protein liquid chromatography (FPLC) or high pressure liquid chromatography (HPLC) and preparative sodium dodecyl sulfate (SDS) gel electrophoresis. This was done to determine the N-terminal amino acid sequencing using electroblotting and gas-phase microsequencing techniques. Two-dimensional gel electrophoresis was used to determine the purity of the preparations and to compare with the established two-dimensional maps of nuclear proteins.

Purification of ets-1 and ets-2 Proteins: Additionally, we have used the monoclonal antibodies to the ets protein to prepare antibody affinity resins. Using these resins and radioimmune precipitation assay (RIPA) solubilized proteins from CEM cells, we have isolated about 1 μg/g (w/w) cells of ets-1 and 0.1 μg/g (w/w) cells of ets-2. The purified proteins are now being used for DNA binding experiments, N-terminal amino acid terminations, and as substrates for phosphorylation reactions.

Protein Sequence Analysis: The ASCL VAX and CRAY supercomputer facility is used to analyze amino sequences of the ets and ets-related proteins.

Major Findings:

Antibodies: Our work with the characterization of the antipeptide antibody to the sequence, FKLSDPDEVARRW, led to the realization that this antibody recognized all of the known ets proteins, including ets-1, ets-2, erg-1, erg-2, and v-ets, as well as other cellular proteins which may be defined as ets-related antigens. Subsequently, a monoclonal to this peptide sequence was developed. This antibody is a pan-ets reagent. Monoclonal antibodies were developed against the bacterially-expressed ets-2 protein and these recognize a subset of the p56 proteins previously identified with the pan-ets antibodies. Similarly, monoclonal antibodies were developed to the bacterially-expressed ets-1 proteins, and these antibodies recognize a subset of proteins (p51, p48, p42, p39) which had previously been identified with the pan-ets monoclonal reagent. Two-dimensional gel electrophoresis of proteins immunoprecipitated from ^{35}S-methionine-labelled COLO 320 or CEM cells allowed the distinction between the ets-related antigens identified with the pan-ets antibody and the ets-2 protein identified with the ets-2 monoclonal antibody.

Characterization of the ets-1 protein: Our new monoclonal antibody to ets-1 identified several more nuclear acidic proteins distinct from ets-2. These proteins are more abundant than the ets-2 proteins and show both nuclear and cytoplasmic localization. Pulse-chase experiments show that there is no precursor product relationship between the non-phosphorylated forms of the ets-1 proteins. We have found a p51 predominantly cytoplasmic protein which can be phosphorylated to a pp52 protein. This protein corresponds to the ets-1 protein previously identified in our laboratory using an antipeptide antibody specific for ets-1. Additionally, a nuclear p48, which can be phosphorylated to a pp49, and two non-phosphorylated p42 and p39 nuclear proteins were found. Peptide mapping of these proteins indicated that they are all related, but that the p42 and p39 lacked several peptides shown to be phosphorylated in the p48 and p51 proteins. Examination of purified ets-1 proteins with exon-specific antipeptide antibodies demonstrated that, while the p51 and p48 reacted with all of the ets-1 exon-specific antibodies, the p39 and p42 did not react with an ets-1 exon 7-specific antibody. This data suggested to us that the p42 and p39 ets-1 proteins may have come from alternatively-spliced mRNA which are lacking exon 7. Examination of the exon 7 amino acid sequence reveals a putative calcium-dependent protein kinase phosphorylation site (RXXS/T). These results are consistent with the calcium-dependent phosphorylation of the ets proteins and suggest that the p39 and p42 are not subject to the same regulation as the p48 and p51.

Characterization of the ets-2 protein: We have shown that the nuclear ets-2 protein is phosphorylated, has a 20 minute half-life, is an acidic protein, and responds to the mitogenic activation of the protein kinase C by increasing its half-life from 20 minutes to greater than two hours. Stimulation of the T-cell antigen receptor by antibodies to the antigen receptor, or with calcium ionophore, allowed a rapid phosphorylation of the ets-2 protein. Thus, ets-2 responds to two cellular signalling systems, one by a calcium-dependent phosphorylation, and the other by increasing the amount of ets-2 by a post-translational mechanism. The interaction of the mitogenic signal transduction pathway suggests that the ets-2 protein is a nuclear regulatory protein with properties similar to those of fos, myc, myb, and p53.

Characterization of ets-related proteins: We have found several ets-related proteins with the pan-ets antibody. These proteins are also nuclear proteins, but are positively charged, and at least a p31 and p37 co-purify with hnRNP proteins. The positively-charged p56 ets-related protein co-extracts with the hnRNP and snRNP proteins, but does not seem to be physically associated with these proteins. The ets-related p56 was isolated and its N-terminal amino acid sequence was determined to be AKDVKFGADARALMQGVDL. A polyclonal antibody to this sequence recognized p56 and p60 nuclear proteins by Western blotting.

Immunohistochemistry of the ets proteins: The pan-ets antibody has been shown to react with the nuclei of COLO 320 cells using biotinylated second antibodies and avidin D-coupled horseradish peroxidase. Examination of cells and tissues with the monoclonal antibodies to pan-ets, ets-1 or ets-2 have, thus far, been negative by either immunofluorescence or immunoperoxidase methods. Using immunoelectron microscopy and second antibodies coupled to 5nm, 10nm or 15nm gold particles, we have been able to show that the ets-1 and ets-2 monoclonal antibodies decorate nuclear fibrils in transcriptionally-active regions of lymphocyte nuclei (CEM cells). Each of these antibodies also react with unidentified cytoplasmic fibrils. In double-labelling experiments, where DNA/ets-1 or DNA/ets-2 were examined, the results show that at least some of the ets proteins are associated with DNA.

Quantitation of the ets proteins: The rapid turnover of the ets-2 protein allowed us to quantitate the ets-2 protein by metabolic labelling with ^{35}S-methionine. Quantitative immunoprecipitations were carried out, and we found that there were about 5000 molecules of ets-2 per CEM cell. Preliminary experiments with ets-1 quantitation show about 10-fold higher ets-1 protein than ets-2 protein.

Publications:

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Fujiwara S, Fisher RJ, Bhat NK, Diaz de la Espina SM, Papas TS. A short-lived nuclear phosphoprotein encoded by human ets-2 proto-oncogene is stabilized by activators of protein kinase C. Mol Cell Biol 1988;8:4700-6.

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Papas TS, Samuel KP, Lautenberger JA, Dubois GC, Zweig M, Showalter SD, Seth A, Fisher RJ, Fujiwara S, Schweinfest CW, Shih T, Ascione R. Vector systems for the high-level expression of oncogenes, HTLV-I and HIV polypeptides. In: Albertini A, Paoletti R, Reisfeld RA, eds. Molecular probes: technical and medical applications. New York: Raven Press, 1989;79-90.

Psallidopoulos MC, Seth A, Dubois GC, Fisher RJ, Papas TS. Expression and characterization of a protein encoded by the human c-myc exon 1 in Escherichia coli. Gene Anal Techn (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05442-05 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Human ETS Genes in Human and Cancer Genetics

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	N. Sacchi	Visiting Scientist	LMO NCI
Others:	T. S. Papas	Chief	LMO NCI
	C. L. Jorcyk	Biologist	LMO NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.14	1.04	0.10

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The members of the ETS gene family now accounting for five independent loci: ETS1, ETS2, ERG, ELK1, ELK2, have been localized at chromosome regions, some of which are of genetic interest, both for constitutional and acquired (neoplasia) genetic disease. We have been focusing on two of these regions, the 11q23 and 21q22 regions, where ETS1 and ETS2 have been mapped. The ETS1 gene has been found (1) transposed, but not structurally altered in several translocations associated with acute non-lymphocytic leukemia (Sacchi et al., Science 1986;231:379-82); and it is transcribed at very low levels in ANLL leukemias (Sacchi et al., Leukemia 1988;2:12-8), but remains normal-sized, regardless of whether it is positive for translocations. (2) It is neither transposed nor structurally involved in Ewing's sarcoma, neuroepithelioma, Askin's tumor with (11;22) translocation or in the constitutional (11;22) chromosomopathy.

The hypothesis of a relationship between ETS2 and Down's syndrome has been tested on two grounds. While ETS2 tested negative as a putative cis-acting genetic element, not interfering with correct chromosome 21 segregation at meiosis (Sacchi et al., Proc. Natl. Acad. Sci. USA 1988;85:4794-8, 1988) it was shown to be a component of the minimal genetic region responsible for Down's syndrome (DS) (Sacchi et al., Proc. Natl. Acad. Sci. USA 1988;85:7675-9). Increased ETS2 gene dosage, certainly extraneous to Alzheimer's disease (AD), is probably not the cause of AD developed by DS individuals. That higher ETS2 gene dosage might be responsible for the post-natal predisposition to leukemia in DS remains to be proven. Nevertheless, we have shown that at least a second gene, different from ETS2 and of relevance to leukemogenesis, must reside on chromosome 21, since the t(8;21) breakpoint of acute myelogenous leukemia M2 is more than 17 cM proximal to ETS2 (Sacchi et al., Genomics 1988;3:110-6). Extension of this work--the introduction of the ETS2 gene in transgenic mice--is reported by A. Seth.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

N. Sacchi	Visiting Scientist	LMO NCI
T. S. Papas	Chief	LMO NCI
C. L. Jorcyk	Biologist	LMO NCI

Objectives:

The regions adjacent to ETS1 and ETS2 will be defined since, as previously outlined, the presence of these genes is not sufficient to explain certain pathologies. The 11q23 region carries a few genes: NCAM, APOA1-4, THY1, T3$\delta\epsilon\gamma$, all involved in cell signaling. All these genes, including ETS1, will be ordered to establish a refined physical and genetic map, in conjunction with DNA polymorphic probes specific for chromosome 11. A refined map is a prerequisite to identify (1) rearrangements of the 11q23 marking numerous acute non-lymphocytic leukemias; (2) an ataxia telangectasia (AT) gene (complementation group A). AT is a condition associated to high incidence of leukemia, and lymphomas; (3) the specificity of 11q deletions in breast cancer since ataxia telangectasia carriers (with one lesion at the AT locus) are more prone to develop cancer, particularly breast cancer in women. In this respect, we plan to investigate a large cohort of patients with breast cancer for 11q23 deletion using a number of chromosome 11 probes. As far as the ETS1 gene is concerned, possible altered ETS1 transcripts will be searched by using a PCR technique much more sensitive than the conventional Northern blot analysis (C. Jorcyk, unpublished results).

The 21q22 region will also continue to be defined, mainly with the objective to identify the t(8;21)(q22;q22) lesion, and improve the DS minimal critical region map.

Methods Employed:

1. Long-range restriction mapping technique.

2. Preparative pulse-field gel electrophoresis and development of new 11q23 21q22 probes.

3. Linkage analysis.

Major Findings:

A major effort has been devoted to the extension of the 11q23 and 21q22 regions, considerably huge from a physical standpoint, measuring at least 40 cM and 20 cM, respectively. Numerous loci (anonymous) have been ordered relative to two sites of special interests to us, the breakpoint of the (4;11) translocation and (8;21) translocation of acute non-lymphocytic leukemias.

Furthermore, a preliminary very interesting result showed deletion of an SstI ETS1 restriction fragment length polymorphism (Sacchi et al., Nucleic Acids Res. 1986;14:9545) in breast cancer tissue, but not peripheral blood lymphocytes of the same individuals, in a limited cohort so far investigated. The availability of markers, proximal and distal to ETS1, will be explored to investigate 11q23 allele losses in sporadic versus premenopausal (inherited?) breast tumors.

Publications:

Cheng SV, Nadeau JH, Tanzi RE, Watkins PC, Sacchi N, Gusella JF. Synteny in man and mouse of DNA markers from the chromosomal region linked to familial Alzheimer's disease and Down syndrome. Proc Natl Acad Sci USA 1988;85:6032-6.

Dagna Bricarelli F, Pierluigi M, Perroni L, Grasso M, Arslanian A, Sacchi N. High efficiency in the attribution of parental origin of nondisjunction in trisomy 21 by both cytogenetic and molecular polymorphisms. Hum Genet 1988;79:124-7.

Papas TS, Blair DG, Fisher RJ, Watson DK, Sacchi N, Fujiwara S, Bhat N, Ascione R. The ets genes. In: Reddy EP, Skalka AM, Curran T, eds. The oncogene handbook. New York: Elsevier, 1988;467-85.

Sacchi N, Cheng SV, Tanzi RE, Gusella JF, Drabkin HA, Patterson D, Haines JH, Papas TS. The ETS genes are distal to the chromosome 21 breakpoint of the acute myelogenous leukemia translocation 8;21. Genomics 1988;3:110-6.

Sacchi N, Nalbantoglu J, Sergovich FR, Papas TS. The ETS2 gene is not rearranged in Alzheimer disease. Proc Natl Acad Sci USA 1988;85:7675-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05443-05 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Proto-Oncogene Expression During Cell Differentiation and Development

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	T. S. Papas	Chief	LMO	NCI
Others:	R. J. Fisher	Expert	LMO	NCI
	S. Fujiwara	Visiting Associate	LMO	NCI
	R. Ascione	Research Chemist	LMO	NCI
	S. Koizumi	Visiting Fellow	LMO	NCI
	L. Bristol	IRTA Fellow	BRMP	NCI
	H. Young	Microbiologist	BRMP	NCI

COOPERATING UNITS (if any)
Nuc. Acid & Prot. Syn. Lab., PRI, Frederick, MD (N. K. Bhat, S. Showalter, M. Zuber, R. Patel); Biological Carcinogenesis and Development Program, PRI, Frederick, MD (K. L. Komschlies, F. Aiello)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.94	0.94	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The murine ets gene products (ets-1 and ets-2) are expressed at higher levels in CD4+ CD8- thymocytes than in CD4+ CD8- T-cells. The effect of mitogens, lymphokines and activators of second messengers (activators of protein kinase C [PKC] and calcium-dependent protein kinase) was studied on isolated peripheral T-cells and in two different murine helper subset cell lines (Th1 and Th2) to dissect the role of ets gene products during T-cell activation and proliferation. The peripheral T-cells express more ets-1 than ets-2 mRNA. When quiescent T-cells are stimulated to proliferate with either cross-linking TCR/CD3 receptors or with a combination of phytohemagglutinin and ionomycin, the ets-2 mRNA is induced by five- to tenfold, whereas the ets-1 mRNA is reduced to basal levels. These results, in conjunction with the appearance of both ets-1 and ets-2 gene products during 3T3 fibroblast cell proliferation, further support the hypothesis that the ets-2 gene products play a role during the early phase of cell proliferation.

Both murine ets-2 and human ERG cDNA clones have been expressed in prokaryotic expression systems in order to generate large amounts of proto-oncogene proteins. These polypeptides enabled us to prepare both monoclonal and polyclonal antisera. Characterization of monoclonal antibodies directed against various oligopeptides derived from the conserved domain of the ets family of genes is underway. Use of the ets and erg gene products and their specific antibodies as potential diagnostic markers for particular types of leukemias is under investigation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. S. Papas	Chief	LMO NCI
R. J. Fisher	Expert	LMO NCI
S. Fujiwara	Visiting Associate	LMO NCI
R. Ascione	Research Chemist	LMO NCI
S. Koizumi	Visiting Fellow	LMO NCI
L. Bristol	IRTA Fellow	BRMP NCI
H. Young	Microbiologist	BRMP NCI

Objectives:

To determine the role of nuclear oncogene products, specifically the role of the ets gene products in cell proliferation and differentiation, and to understand the molecular mechanisms involved in the regulation of ets gene expression. To use probes of the ets genes as potential diagnostic markers for particular types of leukemias. To express ets gene polypeptides to generate antigens for the production of specific antibodies in order to target the authentic cellular products.

Methods Employed:

1. Isolation of nucleic acids, and RNA and DNA blot transfer analyses. Isolation of high molecular weight DNA, total poly A+ RNA, nucleic acid fractionation on agarose gels, transfer of nucleic acids to membranes, probe preparation, hybridization and washing of filters were done as described by Maniatis et al. (Molecular Cloning, A Laboratory Manual, 1982).

2. Cloning. Restriction enzyme digestion, isolation of DNA fragments from gels by electroelution and elutip-d column chromatography, ligation, transformation of competent cells by plasmids, plasmid isolation and characterization were carried out as described by Maniatis et al. Appropriate viral and cellular ets DNA fragments were subcloned in Germini vectors to get a higher yield of plasmids and to prepare labeled riboprobes. The murine ets-2 cDNA, and human ERG-1 and ERG-2 cDNA fragments were expressed in bacteria using expression vector systems available in LMO.

3. Isolation of T-Cells: Murine lymph node T-cells are isolated by a nylon-wool column, as described (Brownell et al., Mol Cell Biol 1987;8:1304-8). The Th-1 and Th-2 type helper T-cell clones were maintained using standard tissue culture techniques. To study the effect of mitogens (concanvalin A, lymphokines IL-1 or IL-2; activators of protein kinase C (PMA), and CAM Kinase, calcium ionophore) on T-cells, optimum doses of the drug have been used. At different time points cells were harvested and RNA was isolated, as described above.

4. Cell labeling, immunoprecipitation and protein blot analyses: These experiments were carried out as described (Fujiwara et al., Mol Cell Biol 1988;8:4700-6).

Major Findings:

1. During the early phase of cell proliferation (within 6 hours), both murine ets-1 and ets-2 gene products accumulate, both at the mRNA and protein levels.

2. During T-cell proliferation by the CD3 activation pathway, ets-1 mRNA expression is down modulated, whereas ets-2 mRNA expression is up regulated; this process appears to be dependent on protein synthesis.

3. Murine ets proteins are phosphorylated by a protein kinase that is activated by Ca^{2+} ions.

4. The ets genes are regulated differently in different cell types. After treatment with protein synthesis inhibitors, ets-2, but not ets-1, mRNA is superinduced in cells that have basal levels of both ets-1 and ets-2 mRNA.

5. Monoclonal antibodies derived against the conserved domain of the ets family of genes react with human ETS-1, ETS-2, ERG-1 and ERG-2 proteins, as expected.

6. The ERG gene expression appears to be higher in carcinoma cells derived from sigmoidal colon tissue. The expression of ERG genes appears to be higher in blast populations derived from acute lymphocytic leukemic patients.

Publications:

Bhat NK, Showalter SD, Fujiwara S, Fisher RJ, Mathieson BJ, Gregorio TA, Young HA, Kasik JW, Ozato K, Papas TS. Expression of ets genes in mouse thymocyte subsets and T cells. J Immunol 1989;142:672-8.

Bhat NK, Showalter SD, Fujiwara S, Fisher RJ, Koizumi S, Papas TS. Regulation of ets genes during cell proliferation and differentiation. In: Rotundo RL, Ahmad R, Bialy H, Black S, Brew K, Chaitin MH, Glaser L, Magleby KL, Neary JT, Van Brunt J, and Whelan WJ, eds. Advances in gene technology: molecular neurobiology and neuropharmacology. Oxford/Washington, DC: IRL Press, 1989;130.

Fisher RJ, Fujiwara S, Bhat NK, Schweinfest CW, Papas TS. c-ets-2 and the mitogenic signal pathway. Modern trends in human leukemia VIII. (In Press)

Fujiwara S, Fisher RJ, Bhat NK, Diaz de la Espina SM, Papas TS. A short-lived nuclear phosphoprotein encoded by the human ETS-2 proto-oncogene is stabilized by activation of protein kinase C. Mol Cell Biol 1988;8:4700-6.

Fujiwara S, Fisher RJ, Koizumi S, Bhat NK, Papas TS. Antibody to the T-cell receptor complex stimulates phosphorylation of the ets-2 protein. In: Rotundo RL, Ahmad F, Bialy H, Black S, Brew K, Chaitin MH, Glaser L, Magleby KL, Neary JT, Van Brunt J, and Whelan WJ, eds. Advances in gene technology: molecular neurobiology and neuropharmacology. Oxford/Washington, DC: IRL Press, 1989;132.

Papas TS, Blair DG, Fisher RJ, Watson DK, Sacchi N, Fujiwara S, Bhat N, Ascione R. The ets genes. In: Reddy EP, Skalka AM, Curran T, eds. The oncogene handbook. New York: Elsevier, 1988;467-85.

Sakamoto S, Mathieson B, Komschlies KL, Bhat NK, Young HA. The methylation state of the T-cell antigen receptor β chain gene in subpopulations of mouse thymocytes. Eur J Immunol (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05484-04 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Proto-oncogene ets in Sea Urchin and Xenopus Laevis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Z. Q. Chen	Visiting Associate	LMO NCI
Others:	L. A. Burdett	ITRA Fellow	LMO NCI
	J. A. Lautenberger	Research Chemist	LMO NCI
	R. Ascione	Research Chemist	LMO NCI
	T. S. Papas	Chief	LMO NCI

COOPERATING UNITS *(if any)*
Nucleic Acid and Protein Synthesis Laboratory, Program Resources, Inc., Frederick, MD (S. Showalter)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Office of the Chief

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.79	1.79	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We characterized the proto-oncogene ets-2 homolog isolated from a Xenopus laevis oocyte cDNA library. The open-reading frame length of the ets-2 sequence is 472 amino acids. The putative initiation and termination codons are co-linear with the homologous human and mouse sequences. The entire cDNA sequence was cloned into the bacterial-expression vector, pJL6, and high-level expression of a non-fusion proto-oncogene encoded protein was obtained. The ets-2 expressed protein is being purified to make antiserum and to study its biochemical properties.

The expression pattern of a 3.2 kb proto-oncogene ets-2 mRNA is typical of a maternal mRNA during oogenesis and embryonic development. The mRNA level (on an oocyte/embryo basis) remains almost constant during oogenesis, and a similar level is maintained from the egg stage through early cleavage.

The ets-2 mRNA was found to be nearly evenly distributed throughout the cytoplasm of the oocytes, and not specifically localized in the animal or vegetal pole. Injection of antisense oligonucleotides into oocytes results in the degradation of the endogenous ets-2 mRNA and blocks germinal vesicle breakdown (GVBD) induced by hormone. Thus, the ets-2 product appears to be required for the meiotic maturation of Xenopus oocytes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Z. Q. Chen	Visiting Associate	LMO NCI
L. A. Burdett	IRTA Fellow	LMO NCI
J. A. Lautenberger	Research Chemist	LMO NCI
R. Ascione	Research Chemist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

To define the role and biological function of the proto-oncogene, ets, in cellular growth and differentiation, as well as its evolutionary relationship from invertebrates to man.

Methods Employed:

DNA manipulation. Southern blot; screening a cDNA library with v-ets and Xenopus laevis segments of ets-2 cDNA as probes; purification and enrichment of the positive clone; restriction enzyme analysis; isolation of DNA fragments from gels by electroelution following agarose electrophoresis; elutip and chromatographic separation; ligation; transformation into competent cells by plasmid constructs; plasmid preparation, isolation and labeled probes were performed as described by Maniatis et al., 1982). Sequencing of DNA was performed by the chain-terminator method using the M13 system (Proc Natl Acad Sci USA 1977;74:5463-7).

RNA manipulation. The isolation and selection of RNA from different stages of embryos, oocytes and eggs; mRNA fractionation and analysis; and Northern blot analyses were performed according to standard procedures (Molecular Cloning, Maniatis et al., 1982).

Protein manipulation. Analysis of ets-2-related protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and specific antibody against ets-2-predicted oligopeptide; by immunoelectrophoresis (Western blot) and immunoprecipitation to identify the ets product during different stages of embryogenesis.

Embryo and microinjection. Isolation of staged oocytes; fertilization in vitro; embryo development and microinjections were performed according to Gurdon and Wicken (Methods Enzymol 1983;101-370) and Dumont (Morph 1972;136:153-64). Oocyte sectioning was as described by Melton (Cell 1985;42:769-77).

Major Findings:

We have identified a homolog of Xenopus laevis cDNA with the v-ets-2 gene. The full-length sequence encoded 472 amino acids; we cloned the entire Xenopus ets-cDNA sequence into the pJL6 expression vector and obtained high-level expression of a non-fusion proto-ets-2 protein. The in situ analysis

of oocytes revealed that ets-2 mRNA was to be nearly evenly distributed throughout the cytoplasm of oocytes and not specifically localized in the animal or vegetal pole. The expression pattern of the 3.2 kb ets-2 mRNA is typical for maternal mRNA transcription during oogenesis and embryonic development. The mRNA level (on an oocyte/embryo basis) remains almost constant throughout oogenesis and a similar level is maintained during the egg stage through early cleavage. It seems that ets-2 expression is a requirement for Xenopus oocyte maturation. It appears injection of antisense oligonucleotides into oocytes causes the degradation of endogenous ets-2 mRNA and blocks germinal vesicle breakdown (GVBD) inducible by hormone.

Publications:

Chen ZQ, Fujiwara S, Bhat NK, Lautenberger JA, Fisher RJ. Immunological detection of protein antigens after phenol-chloroform denaturation. Gene Anal Techn 1989;4:44-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05485-04 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less Title must fit on one line between the borders.)

Application of Monoclonal Antibodies to the Study of Oncogene Products

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator) (Name, title, laboratory, and institute affiliation)

PI:	S. Fujiwara	Visiting Associate	LMO NCI
Others:	R. J. Fisher	Expert	LMO NCI
	S. Koizumi	Visiting Fellow	LMO NCI
	T. S. Papas	Chief	LMO NCI

COOPERATING UNITS (if any)

Nucleic Acid and Protein Synthesis Laboratory, Program Resources, Inc., Frederick, MD (N.K. Bhat), Centro de la Investigacions, Madrid, Spain (S.M. Diaz de la Espina)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Transgenic Analysis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.59	0.59	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

Monoclonal antibodies facilitate biochemical analysis of proteins by its highly-specific recognition of a single epitope and its availability in large amounts. We are isolating monoclonal antibodies to the proteins encoded by various human proto-oncogenes. Thus far, antibodies against the myc and ets-2 proteins have been produced and are being used to investigate cellular function(s). Recently, we prepared a new monoclonal antibody specific to the proteins encoded by the ets-1 gene, a cellular homolog of the v-ets oncogene carried by the avian leukemia virus, E26. This antibody recognizes multiple forms of the human ets-1 protein (pp52, p51, pp49, p48, p42, and p39). Their primary localization is the nucleus, although a fraction of the proteins were also found in the cytoplasm. Alternative splicing could be partly responsible for the generation of these multiple ets-1 proteins, because the p42 and p39 specifically lack the epitope on exon 7 of the human ets-1 gene. Differential pp52 and pp49 are the phosphorylated forms of p51 and p48, respectively. p42 mitogenic signals to T lymphocytes. Treatment of the human T-cells' Jurkat with various mitogens induced rapid phosphorylation of the ets-1 proteins by a calcium-dependent mechanism. Experiments using another monoclonal antibody gave similar results with the proteins encoded by the ets-2 gene, an ets-1-related gene. The ets-2 protein is also heterogenous, consisting of two components, p56 and p54. Phosphorylation of the ets-2 proteins was also stimulated by T-cell mitogens in a calcium-dependent manner. These results suggest that the ets-1 and ets-2 proteins have a role in the calcium-mediated signal transduction in T lymphocytes. Their nuclear localization and DNA binding activity are consistent with the possibility that they take part in gene regulation linked to T-cell activation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

S. Fujiwara	Visiting Associate	LMO NCI
R. J. Fisher	Expert	LMO NCI
S. Koizumi	Visiting Fellow	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

The immediate objective of this project is to generate highly-specific immunological probes for the proteins encoded by human proto-oncogenes. These probes are to be applied to biological and biochemical characterization of the proteins in search of their normal functions. As a specific probe for a single epitope, monoclonal antibodies are particularly useful in investigating protein structure at the individual domain level. Detailed analysis of the structure of the oncoproteins performed in relation to functional parameters should give insight into the molecular mechanisms by which these proteins mediate malignant cellular transformation. The monoclonal antibodies will be used as probes in various basic experiments involving immunochemical detection of the oncoproteins. In addition, monoclonal antibodies have the potential to be used as the diagnostics for the clinical conditions associated with abnormal expression of the oncoproteins.

Methods Employed:

The antigens used in hybridoma preparation were produced in E. coli from the cDNAs of the proto-oncogenes cloned into bacterial expression vectors. BALB/c mice were immunized with these antigens and hybridomas were generated using standard procedures, including polyethylene glycol-mediated cell fusion and hypoxanthine-aminopterine-thymidine (HAT) selection of hybrid cells. Hybridoma culture fluids were screened for specific antibodies by the enzyme-linked immunosorbent assay (ELISA). The proteins reactive with the monoclonal antibodies were analyzed by immunoprecipitation and immunoblot assay, combined with one-dimensional or two-dimensional gel electrophoresis. The antigens were also detected "in situ" by immunocytochemical methods followed by light or electron microscopy. Structural characterization of the proteins were done by peptide mapping using various proteolytic enzymes. Immunoaffinity chromatography was used to purify the proteins reactive with the monoclonal antibodies.

Major Findings:

This year, we have prepared a new monoclonal antibody specific to the ets-1 protein. This antibody recognized multiple species of the human ets-1 protein, namely, pp52, p51, pp49, p48, p42 and p39. Reactivity of these proteins with three other independent ets-1 antibodies and structural analysis by peptide mapping indicated that they are all products of the human ets-1 gene. The p42 and p39 did not react with the antibody directed to an epitope

on the exon 7 of the human ets-1 gene, suggesting that they are the products of the mRNA which lacks this exon as the result of alternative splicing. The multiple forms of the ets-1 proteins seem to have different localization in the cell. In subcellular fractionation, the p52 and p51 were cytoplasmic, the p48 and p39 were nuclear, and the p49 and p42 were distributed between cytoplasm and nucleus.

Consistent with these results, immunoelectron microscopy detected the ets-1 protein in both the cytoplasm and nucleus. The ets-1 proteins were found differentially phosphorylated; the p52 and p49 are the phosphorylated forms of the p51 and p48, respectively. The p42 and p39 are not phosphorylated. The phosphorylation of the ets-1 proteins was quickly stimulated (within five minutes) by treating the cells with T-cell mitogens or co-mitogens, such as antibodies against the T-cell antigen receptor/CD3 complex or plant lectins. This phosphorylation is probably mediated by Ca^{2+}, because the mitogens used are known to increase the cytoplasmic Ca^{2+} concentration, and Ca^{2+} ionophores also induced phosphorylation of the ets-1 proteins. Basically, similar results were obtained for the human ets-2 proteins by using an ets-2-specific monoclonal antibody. The human ets-2 protein consists of two components, p56 and p54; the former is the phosphorylated form of the latter. Phosphorylation of the p54 into p56 is stimulated by mitogenic signals for the T lymphocytes in a Ca^{2+}-dependent manner.

Publications:

Bhat NK, Komschlies KL, Fujiwara S, Fisher RJ, Mathieson BJ, Gregorio TA, Young JA, Kasik JW, Ozato K, Papas TS. Expression of ets genes in mouse thymocyte subsets and T cells. J Immunol 1989;142:672-8.

Bhat NK, Showalter SD, Fujiwara S, Fisher RJ, Koizumi S, Papas TS. Regulation of ets genes during cell proliferation and differentiation. In: Rotundo RL, Ahmad F, Bialy H, Black S, Brew K, Chaitin MH, Glaser JL, Magleby KL, Neary JT, Van Brunt J, Whelan WJ, eds. Advances in gene technology: molecular neurobiology and neuropharmacology. Oxford/Washington DC: IRL Press, 1989;130.

Chen ZQ, Fujiwara S, Bhat NK, Fisher RJ. Immunological detection of protein antigens after phenol-chloroform denaturation. Gene Anal Techn 1989;6:44-6.

Fisher RJ, Fujiwara S, Bhat NK, Schweinfest CW, Papas TS. c-ets-2 and the mitogenic signal pathway. In: Modern trends in human leukemia VIII. Berlin: Springer-Verlag (In Press)

Fujiwara S, Fisher RJ, Bhat NK, Diaz de la Espina SM, Papas TS. A short-lived nuclear phosphoprotein encoded by the human ets-2 proto-oncogene is stabilized by activation of protein kinase C. Mol Cell Biol 1988;8:4700-6.

Fujiwara S, Koizumi S, Fisher RJ, Bhat NK, Papas TS. Antibody to the T-cell receptor complex stimulates phosphorylation of the ets-2 protein. In: Rotundo RL, Ahmad F, Bialy H, Black S, Brew K, Chaitin MH, Glaser L, Magleby KL, Neary JT, Van Brunt J and Whelan WJ, eds. Advances in gene technology: molecular neurobiology and neuropharmacology. Oxford/Washington DC: IRL Press, 1989;132.

Papas TS, Blair DG, Fisher RJ, Watson DK, Sacchi N, Fujiwara S, Bhat NK, Ascione R. The ets genes. In: Reddy EP, Skalka A, Curran T, eds. The oncogene handbook. New York: Elsevier, 1988;467-555.

Papas TS, Samuel KP, Lautenberger JA, Dubois GC, Zweig M, Showalter SD, Seth A, Fisher RJ, Fujiwara S, Schweinfest CW, Shih T, Ascione R. Vector systems for the high-level expression of oncogenes, HTLV-I and HIV polypeptides. In: Albertini A, Paoletti R, Reisfeld RA, eds. Molecular probes: technical and medical applications. New York: Raven Press, 1989;79-90.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05563-02 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Introduction of the HIV tat Gene into Lymphoid Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. A. Lautenberger	Research Chemist	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*
Bionetics Research, Inc., Frederick, MD (B. Felber, G. Pavlakis)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.19	0.19	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In order to study the effect of the human immunodeficiency virus (HIV) tat gene on the expression of cellular genes, we have constructed lymphoid lines that expressed the tat gene. This was done by placing the tat gene and an adjacent HIV long terminal repeat (LTR) into the retroviral vector pGV1. The recombinant plasmid was transfected into psi2 cells to produce an ecotropic viral stock that was used to infect psiAM cells. Colonies of G418-resistant psiAM cells were isolated and assayed for the production of virus. Supernatants of those colonies with the highest virus production were used to infect cells of the human T-cell lymphoid line, H9. G418-resistant cell lines derived from H9 were found to contain tat activity as assayed by fusion with HeLa cells containing LTR and CAT sequences. Furthermore, they contained an LTR-tat message of the predicted size.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. A. Lautenberger	Research Chemist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

The HIV tat gene has been shown to regulate transcription initiated at the viral LTR and possibly regulate translation of viral proteins. Since tat has not been shown to bind HIV DNA, this protein may induce the expression of cellular genes. We plan to investigate this possibility by the methods of differential hybridization and subtractive hybridization. Each of these methods require RNA from both tat^+ and tat^- cells that are otherwise isogenic. For these purposes, it is also desirable that the cells used be of lymphoid origin since $CD4^+$ lymphocytes are a major target of HIV. Because it is difficult to introduce DNA into lymphocytes by many of the conventional techniques, we have chosen to use recombinant retroviruses to achieve this goal.

Methods Employed:

1. Construction of recombinant plasmids. Plasmid pMVL3ctat was digested with restriction enzymes, Asp718I and BamHI, and fragments were resolved by agarose gel electrophoresis. An 840 bp fragment containing the HIV-1 LTR and tat sequences was isolated from the gel by the glass bead absorption method (Bio101, Inc.). The ends of this fragment were made blunt by filling them in with Klenow polymerase and the fragment was ligated to BamHI linkers. The ligation reaction product was digested with BamHI to remove excess linkers, and the fragment, now containing BamHI termini, was repurified by agarose gel electrophoresis. This DNA was ligated to plasmid pGV1 DNA that had been linearized by digestion with BamHI. Transformation competent Escherichia coli strain HB101 cells (BRL) were transformed by the ligation reaction product and selected for kanamycin resistance. Resistant colonies were screened for the presence of recombinant plasmids by restriction enzyme analysis of small-scale plasmid preparations.

2. Isolation of human lymphoid cell line expressing HIV-1 tat. Plasmid pGV1-LTR-tat was transfected into cell line, psi2 (Mann et al., Cell 1983;33:153), which contains a packaging-defective murine sarcoma virus (MSV). Forty-eight hours after transfection, the medium of the psi2 cells containing recombinant helper-free retrovirus was collected and was used to infect psiAM cells, which contain a packaging-defective MSV retrovirus carrying an amphotropic env coat

(Cone and Mulligan, Proc Natl Acad Sci USA 1984;81:6349), allowing infection of human cells. The infected psiAM cells were treated with G418, and resistant psiAM colonies containing integrated proviruses were tested for virus production on HeLa cells. Supernatants from the colonies that produced high levels of virus were used to infect human T-cell line H9 cells. Infected cells were identified by G418 resistance.

3. Cell fusion assay. HL3T1 (Wright et al., Science 1986;234:988) is a derivative of HeLa that contains a chloramphenicol acetyl transferase (CAT) gene under the transcriptional control of the HIV LTR promoter. Candidate tat-producing cells were mixed with HL3T1 cells, washed, and resuspended in PEG solution (50% w/v PEG in RPMI medium) for 3 min., centrifuged for 3 min. at 1200 rpm, washed, and grown for 2 days in RPMI + 10% fetal calf serum. After two days in culture the activation of HIV LTR is measured by assaying for CAT activity.

4. S1 assay. Total RNA was extracted from candidate tat-producing cells and hybridized to a uniformly-labelled, single-stranded DNA probe spanning the transcriptional start site of the HIV LTR. After treatment with S1 nuclease, the protected fragments were resolved on a 6% acrylamide - 8M urea gel.

Major Findings:

Infection of H9 cells with the amphotropic recombinant virus gave rise to G418-resistant colonies. Cells grown from these colonies contained a message that gave the size expected on an S1 nuclease protection assay for a transcript initiating on the HIV LTR and running into the tat sequences. When these cells were fused with HL3T1 cells, extracts of the fused cells were found to have CAT activity. This indicates that the infected H9 cells contain a functional tat gene that is expressed using the HIV LTR as a promoter.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05564-02 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Analysis of HIV Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. A. Lautenberger	Research Chemist	LMO NCI
Others:	C. W. Schweinfest	Staff Fellow	LMO NCI
	T. S. Papas	Chief	LMO NCI

COOPERATING UNITS *(if any)*
Walter Reed Army Institute of Research, Silver Spring, MD (H.E. Gendelman)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.29	0.29	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The expression of human immunodeficiency virus type 1 (HIV-1) in a variety of infected cells was studied by RNA gel blotting. The highest levels of expression were seen in human T lymphotropic virus (HTLV)-III-infected H9 cells and fresh peripheral blood lymphocytes (PBLs). Other strains of the virus were expressed in PBLs at a lower level. Virus-specific messages were detected in infected macrophages, but at a somewhat lower level than in PBLs, even when the virus analyzed was a macrophage-tropic strain. cDNA libraries have been constructed from infected and uninfected macrophages so as to allow the isolation of cellular sequences that are expressed at higher levels in infected cells. These sequences will be enriched for by cDNA library subtraction and will be identified by differential hybridization.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. A. Lautenberger	Research Chemist	LMO	NCI
C. W. Schweinfest	Staff Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

In order to develop methods of controlling human immunodeficiency virus (HIV), it will be necessary to understand its biological variability and its mode of expression in different human cells. It is especially important to study the monocyte/macrophage as a host, since these cells may be a reservoir for the virus. The study of cellular genes that are modulated by the virus may yield markers that are useful in controlling the infection. This is important since viral markers cannot be detected on the surface of infected macrophages.

Methods Employed:

1. RNA gel blot analysis. RNA is prepared by disrupting cells by the method of Chirgwin et al. (Biochem 1979;18:5294-9) and purified by sedimentation through 5.7 M CsCl (Glisin et al., Biochem 1973;13:2633-7). The RNA is transferred by capillary blotting to NYTRAN membranes. The filters are hybridized to radiolabelled HIV sequences in 40% formamide/5XSSC/5X Denhardt's solution/0.5% SDS/0.1 mg/ml Salmon sperm DNA/0.34% nonfat dry milk (1XSSC= 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1X Denhardt's solution=0.02% each BSA, polyvinylpyrrolidone, and Ficoll) and extensively washed. HIV hybridizing sequences are visualized by autoradiography. The radiolabelled HIV probes were generated by the random sequence oligonucleotide primer method of Feinberg and Vogelstein (Anal Biochem 1984;137:266-7).

2. cDNA library construction. cDNA libraries were constructed using the vector lambda ZAP II (Stratagene, Inc.) by published procedures (Gubler and Hoffman, Gene 1983;5:263-9; Okayama and Berg, Mol Cell Biol 1982;2:162-70). The first strand was synthesized with reverse transcriptase using random oligonucleotides and oligo (dT) as a primer, and the second strand was synthesized with a combination of Escherichia coli polI, RNase H, and E. coli DNA ligase. The double-stranded cDNA was ligated to EcoRI adapters. After removal of excess adapters by use of a Sephacryl S-300 spin column, the DNA was ligated to the vector arms and packaged by a commercially available packaging extract (Gigapack Gold from Stratagene, Inc.). The representation of radiolabelled phage in the library was reduced by performing one round of amplification.

3. cDNA library subtraction. A single-stranded cDNA library was prepared by coinfecting Escherichia coli XL-1 blue cells with lambda ZAP II library and an M13 derived helper. Lambda ZAP II is converted to the phagmid bluescript by in vivo excision that is induced by this helper virus. The supernatant of this reaction was used to produce a large-scale phagmid DNA preparation by another round of infection of XL-1 blue. For subtraction, single-stranded DNA

derived from the library of uninfected cells is biotinylated using photoprobe biotin under a sunlamp. The biotinylated DNA is hybridized to single-stranded DNA from a cDNA library derived from infected cells. Common sequences hybridize to the biotinylated DNA. These complexes, as well as unhybridized biotinylated DNA, are reacted to stretavidin and removed by phenol extraction. This occurs because the DNA-biotin-stretavidin complex partitions into the phenol phase, while unhybridized DNA remains in the aqueous phase. The single-stranded DNA in the aqueous phase that has been depleted of common sequences and, thus, is enriched for infection-specific sequences, is converted to double-stranded DNA by reverse transcriptase. This DNA is used to transform XL-1 blue cells. Infection-specific messages are isolated by differential hybridization using probes that are reverse transcribed from RNA from infected cells and from uninfected cells.

Major Findings:

The expression of human immunodeficiency virus type 1 (HIV-1) in a variety of infected cells was studied by RNA gel blotting. The highest levels of expression were seen in HTLV-III-infected H9 cells and fresh peripheral blood lymphocytes (PBLs). Other strains of the virus were expressed in PBLs at a lower level. Virus-specific messages were detected in infected macrophages, but at a somewhat lower level than in PBLs, even when the virus analyzed was a macrophage-tropic strain.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05565-02 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Study of the Biochemical and Functional Properties of the ets Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Seth	Visiting Scientist	LMO	NCI
Others:	D. Thompson	Biologist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Transgenic Analysis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, MD 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.54	0.19	0.35

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ets-1 (p51) and ets-2 (p56)-related proteins have been identified in the human cell lines, Daudi and COLO 320DM, respectively. The p51 is localized in the cytoplasmic and nuclear fractions, while the p56 is present primarily in the nuclear fraction. In spite of this knowledge regarding subcellular localization, the functional role of ets-1 and ets-2 proteins remains to be elucidated.

To study the biochemical properties and also to produce ets-specific antiserum, we have previously constructed several expression vectors capable of producing large quantities of proteins encoded by complete v-ets, partial ets-2 (human) and partial ets-1 (human) and their deletion mutant products in E. coli.

We have constructed a vector (pNAE-9) that expresses the full length protein encoded by the ets-2 gene. The E. coli expressed proteins were tested for their ability to bind nucleic acids; it was observed that v-ets and ets-2 proteins bind DNA when assayed in a nitrocellulose blot protein-DNA binding assay. The ets-2 and ets-1 protein products were used to generate monoclonal antiserum in mice. A panel of monoclonal antibodies were obtained and tested for their ability to recognize p56 (ets-2) and p51 (ets-1) by immunoprecipitations and Western blot analysis. As expected, the ets-1 and ets-2 monoclonals recognize p51-ets-1 and p56-ets-2 products, respectively. Interestingly, the ets-1 and ets-2 monoclonals react only with the human ets-1 and ets-2 products, and do not react with other ets-related gene products. Thus, such monoclonal antibodies should prove very useful in studies on the biochemical properties and the functional role(s) of the ets-1 and ets-2 gene products.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

A. Seth	Visiting Scientist	LMO NCI
D. Thompson	Biologist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

Expression of the viral and human ets-1 and ets-2 proto-oncogene products in E. coli.

Methods Employed:

The restriction enzyme digestions, gel electrophoresis, isolation of DNA insert from gels by electroelution, ligation of insert to vector DNA, bacterial transformation, screening of clones by in situ colony hybridization, DNA probes by nick-translation, double-strand DNA sequencing, small-scale DNA preparation and analysis of the DNA by gel electrophoresis were according to published procedures.

The procedures for thermal induction of proteins, purification of bacterially-expressed proteins, sodium dodecyl sulfate (SDS)-gel electrophoresis, protein labeling with ^{35}S-methionine, protein extraction, immunoprecipitation, Western blotting and production of monoclonal and polyclonal antibodies have been described elsewhere. DNA binding assays were performed on a nitrocellulose blot as described previously. A ^{32}P-labeled, nick-translated ets probe was used as a source of labeled DNA.

Major Findings:

1. Construction of a full-length ets-2 expression vector, pNAE-9. A vector (pNAE-9) that expresses a full-length ets-2 (human) gene product was constructed by insertion of ets-2 cDNA in a previously described vector, pANH1.

2. Expression of a full-length ets-2 product. The bacterially-expressed ets-2 product was characterized by Western blot and immunoprecipitation analysis using ets-specific monoclonal and polyclonal antibodies.

3. ets proteins bind DNA. The purified ets-2 and v-ets proteins were shown to bind DNA when tested in a nitrocellulose blot protein DNA binding assay.

4. Monoclonal antibodies directed against bacterially-expressed ets proteins are highly specific. ets-1 and ets-2-specific monclonal antibodies were obtained in mice. These monclonals are highly specific and react only with the $p51^{ets-1}$ and $p56^{ets-2}$ products, respectively. The availability of such monoclonals will allow us to study the functions of ets-1 and ets-2 proteins in normal and transformed cells.

Publications:

Gonzatti-Haces M, Seth A, Park M, Copeland R, Oroszlan S, Vande Woude GF. Characterization of the tpr-met oncogene p65 and the met proto-oncogene cell surface p140 tyrosine kinase. Proc Natl Acad Sci USA 1988;85:21-5.

Lautenberger JA, DuBois GC, Samuel KP, Seth A, Showalter SD, Schweinfest CW, Zweig M, Papas TS. Expression of human retroviral proteins in a heterologous system. In: Groopman JE, Chen I, Essex M, Weiss R, eds. Early human retroviruses, UCLA Symposia on Molecular and Cellular Biology, New Series, vol. 119. New York: Alan R. Liss, Inc. (In Press)

Papas TS, Samuel KP, Lautenberger JA, DuBois GC, Zweig M, Showalter SD, Seth A, Fisher RJ, Fujiwara S, Schweinfest C, Shih TY, Ascione R. Vector system for the high-level expression of oncogenes, HTLV-I, and HIV polypeptides. In: Albertini A, Raoletti R, Reisfeld RA, eds. Proceedings of the international symposium on molecular probes: technology and medical applications, New York: Raven Press, 1989;79-90.

Psallidopoulos M, Seth A, DuBois G, Papas TS. Expression of the first exon open reading frame of human myc in E. coli as a fusion protein with v-mos oncogene product. Gene Anal Techn (In Press)

Seth A, Konopka A, Vande Woude GF. Localization of nucleic acid binding domain in mos. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ and Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington DC: IRL Press, 1988;82.

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Seth A, Vande Woude GF. The mos oncogene. In: Reddy EP, Skalka A, Curran T, eds. The oncogene handbook. New York: Elsevier, 1988;195-211.

Seth A, Watson DK, Blair DG, Papas TS. C-ets-2 proto-oncogene has mitogenic and oncogenic activity. Proc Natl Acad Sci USA (In Press)

Seth A, Watson DK, Blair DG, Thompson D, Dunn KJ, Papas TS. C-ets-2, a member of Down syndrome locus, has mitogenic and oncogenic activity. In: Rotundo RL, Ahmad F, Bialy H, Black S, Brew K, Chaitin MH, Glaser L, Magleby KL, Neary JT, Van Brunt J, Whelan WJ, eds. Advance in gene technology: molecular neurobiology and neuropharmacology. Oxford/Washington DC: IRL Press, 1989;155.

Seth A, Watson DK, Fujiwara S, Showalter SD, Papas TS. Expression of human and viral ets genes in E. coli production of human ets-2 specific monoclonal antibodies. Oncogene Res (In Press)

Wang LC, Gao C, Vaso WC, Seth A, Chen K. The role of v-mos in transformation, oncogenicity and metastatic potential of mink lung cells. Oncogene 1988;3: 267-73.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05566-02 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Study of the Biological & Biochemical Function of ets Proto-oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Seth	Visiting Scientist	LMO	NCI
Others:	D. Thompson	Biologist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI
	D. G. Blair	Supv. Research Chemist	LMO	NCI

COOPERATING UNITS *(if any)*

Nucleic Acid & Protein Synthesis Laboratory, Program Resources, Inc. Frederick, MD (J. Olson)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Transgenic Analysis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.69	0.34	0.35

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The c-ets-1 gene is expressed primarily in thymic cells, while the ets-2 gene is expressed ubiquitously. Previous data suggests that the c-ets-2 is a member of the nuclear oncogene family (myc, fos, myb, and p53), a group whose protein products have been linked with cell proliferation and differentiation. However, no biological function for any cellular ets genes have, thus far, been described. In an effort to study the role of the ets proto-oncogenes in cell proliferation and transformation, we have constructed vectors containing either the ets-1 or ets-2 proto-oncogene linked to the mouse metallothionein I promoter. NIH 3T3 cells transfected with either construct showed foci of densely-growing, morphologically-altered cells when cultured in low-serum (0.05%) or serum-free medium. Control cells transfected with vectors alone did not grow in serum-free medium and the growth was slow in medium containing 0.05% serum. In order to test whether the growth alterations observed with c-ets-1 or c-ets-2 transfected cells are mediated by the expression of ets-1 and ets-2 genes, we have cloned and analyzed several foci for the presence and expression of metallothionein-linked ets genes. All foci examined contained multiple copies of the expected ets gene and high-level expression of the appropriate ets-specific mRNA. Protein extracts prepared from ets-2-derived cell lines show a high level of the 56Kd ets-2 product. To test whether the expression of high levels of the ets-2 gene in these lines has transforming activity, we analyzed the growth of these lines in soft agar; the results show that the ets-2-transfected cells form colonies in semi-solid media and induce tumors in nude mice. A similar analysis of the ets-1-derived cell lines is in progress. Our data represents a useful assay system to study the role of ets and related genes in cell proliferation and transformation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. Seth	Visiting Scientist	LMO	NCI
D. Thompson	Biologist	LMO	NCI
T. S. Papas	Chief	LMO	NCI
D. G. Blair	Supv. Research Chemist	LMO	NCI

Objectives:

Transforming and mitogenic properties of the c-ets-1 and c-ets-2 proto-oncogenes.

Methods Employed:

The procedures for restriction enzyme digestions, gel electrophoresis, isolation of DNA insert from gels by electroelution, ligation of insert to vector DNA, bacterial transformation, screening of clones by in situ colony hybridization, DNA probes by nick-translation, small-scale DNA preparation and analysis of the DNA by gel electrophoresis have been published.

The DNA transfection of NIH 3T3 cells by the calcium phosphate precipitation procedure, single cell cloning, growth in soft agar, growth in low-serum and serum-free medium, nude mice assay, high molecular DNA extraction, RNA extraction, Southern blots and Northern blots; DNA and RNA blot hybridizations were according to the published procedures.

Protein labeling with [^{35}S]-methionine, protein extraction, immunoprecipitation, and Western blotting with antibodies and [^{125}I] protein have been described previously.

Major Findings:

1. Construction of mammalian expression vectors containing ets-1 and ets-2 proto-oncogene linked to mouse metallothionein promoter and deletion mutants of ets-2 proto-oncogene.

2. Multiple copies of the ets-1 and ets-2 proto-oncogenes. The NIH 3T3 cells were transfected with ets-1 and ets-2 genes carrying vectors, and the cell lines carrying multiple copies of the ets-1 or ets-2, respectively, were further analyzed.

3. ets-1 and ets-2 proto-oncogenes have mitogenic properties. The mouse fibroblasts transfected with either ets-1 or ets-2 showed foci of morphologically-altered cells when grown either in low-serum (0.05%) or serum-free medium. In contrast, the control cells were unable to grow in serum-free medium and did not form foci in medium containing low serum.

4. The ets-1 and ets-2 proto-oncogenes have transforming activity. (a) The ets-1 and ets-2 transfected cell lines were examined for their ability to grow in soft agar suspension. Results show that more than 5% of the transfected cells, either with ets-1 or ets-2, gave rise to colonies in soft agar in two weeks. (b) Tumorgenicity assay. The ets-1 and ets-2-derived cell lines were inoculated into athymic nu/nu mice and examined for tumor development. Both the ets-1 and ets-2 cell lines induced tumors in four weeks. The analysis of tumors by Northern blots revealed they express high levels of ets-2 RNA. A similar analysis with ets-1 tumors is in progress.

5. ets-2 protein is expressed at high levels. In order to test whether the growth alterations observed with c-ets-2-transfected cells are mediated by the expression of the ets-2 gene, we have cloned several foci for ets protein expression. Protein extracts prepared from ets-2-transfected cells show a high level expression of the 56Kd ets-2 product that is not present in control cells. Since the mouse ets-2 contains a consensus sequence for N-linked glycosylation, we are testing whether this protein is glycosylated in vivo.

Publications:

Gonzatti-Haces M, Seth A, Park M, Copeland R, Oroszlan S, Vande Woude GF. Characterization of the tpr-met oncogene p65 and the met proto-oncogene cell surface p140 tyrosine kinase. Proc Natl Acad Sci USA 1988;85:21-5.

Lautenberger JA, DuBois GC, Samuel KP, Seth A, Showalter SD, Schweinfest CW, Zweig M, Papas TS. Expression of human retroviral proteins in a heterologous system. In: Groopman JE, Chen I, Essex M, Weiss R, eds. Early human retroviruses, UCLA Symposia on Molecular and Cellular Biology, New Series, vol. 119. New York: Alan R Liss, Inc (In Press)

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Seth A, Watson DK, Fujiwara S, Showalter SD, Papas TS. Expression of human and viral ets genes in E. coli production of human ets-2-specific monoclonal antibodies. Oncogene Res (In Press)

Wang LC, Gao C, Vaso WC, Seth A, Chen K. The role of v-mos in transformation, oncogenicity and metastatic potential of mink lung cells. Oncogene 1988;3: 267-73.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05569-02 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Effect of c-myc on Cellular Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. W. Schweinfest	Staff Fellow	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI
	S. Fujiwara	Visiting Associate	LMO	NCI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.24	0.24	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A key role for the c-myc oncogene in cellular proliferation has long been postulated. C-myc may act by modulating the expression of other cellular genes whose products directly control proliferation. Permanent cell lines (in which the endogenous c-myc gene is tightly regulated by growth factors and cell/cell contact) have been constructed in which expression of an exogenously transfected c-myc gene is controlled by the Drosophila heat shock 70 promoter. Transcription and subsequent translation of the exogenous c-myc gene is specifically induced by mild heat shock. The endogenous c-myc gene is not expressed under these conditions. Compared to heat-shocked cell lines which contain constructs lacking c-myc, several changes in cellular gene expression are observed: (1) two-dimensional analysis of the proteins from c-myc-containing cell lines shows the induction of eight protein species and the repression of five protein species relative to cell lines lacking c-myc; (2) the transcripts of two genes (3CH77 and 3CH92), previously identified as serum inducible, are induced when c-myc is expressed; and (3) the endogenous heat shock 70 gene may be specifically induced in response to c-myc. Therefore, c-myc expression alters the expression of other cellular genes, including the induction of some known to be expressed only in proliferating cells.

In order to isolate other genes specifically induced or repressed in conjunction with c-myc expression, cDNA libraries have been constructed from which hybridization subtraction may be performed and subtractive libraries made. Subtractive libraries should be enriched for specifically-induced or specifically-repressed sequences.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. W. Schweinfest	Staff Fellow	LMO NCI
T. S. Papas	Chief	LMO NCI
S. Fujiwara	Visiting Associate	LMO NCI

Objectives:

A large body of circumstantial evidence has implicated c-myc as having a role in cellular proliferation in both normal and neoplastic cells. Like most oncogenes, a specific biological function for c-myc has yet to be defined. Since c-myc is one of a class of nuclear oncoproteins and has been demonstrated to have DNA binding capability (albeit nonspecific), c-myc's biological function may be to regulate the expression of other genes. Therefore, the objective of this work is to study the effect of c-myc on the regulation of other genes and, by understanding the function of these target genes, be able to define the role of c-myc.

Methods Employed:

1. A set of cell lines has been constructed in which the human c-myc gene (exons 2 and 3 only, and exons 1, 2 and 3) can be expressed under control of the Drosophila heat shock 70 promoter. Permanent cell lines were constructed in Balb/c 3T3 cells by co-transfection of the myc-containing plasmids, along with the selectable G418-resistance plasmid, pSV2neo, followed by selection in 400 μg/ml G418. Resistant clones are screened for heat shock-inducible expression of c-myc. A control cell line was transfected with a construct lacking the c-myc gene. Under conditions of serum arrest, the endogenous c-myc gene is not expressed.

2. Protein from c-myc-expressing cell lines are analyzed by two-dimensional gel electrophoresis. Non-expressing cell lines are similarly analyzed.

3. The expression of G_0/G_1 transition genes, as well as an S-phase specific gene is assayed as a function or specific c-myc induction by Northern blot hybridization analysis.

4. New genes, whose expression may be modulated by c-myc, are screened for by differential hybridization of cDNA libraries constructed from heat shock-induced, myc-expressing cell lines.

5. Hybridization subtraction will be performed to make subtraction libraries enriched for specifically-induced or specifically-repressed sequences.

Major Findings:

1. Exogenously-transfected human c-myc can be expressed under control of the Drosophila heat shock 70 promoter.

2. Two-dimensional gel electrophoresis, which compares proteins from induced vs. uninduced cells (as well as heat shock-induced negative control cells) reveals the induction of eight protein species and the repression of five protein species.

3. Two genes (3CH77 and 3CH92), previously identified as transcriptionally induced during proliferation, are induced in response to c-myc expression. In response to growth factors, these genes are expressed prior to myc (which also responds to growth factors). However, c-myc expression in the absence of added growth factors precedes expression of 3CH77 and 3CH92. Expression of an S-phase gene upon c-myc induction is not observed.

4. The endogenous heat shock gene itself may be expressed at even more elevated levels in heat shock cells expressing c-myc than those lacking c-myc. A cDNA clone, #32, which is found to be induced by c-myc-expressing cells, hybridizes with a known hsp70 probe.

5. The cDNA library from c-myc-expressing cells has also been used to find a completely coded mouse ets-2 cDNA (see Project Z01CP05238-08 LMO).

6. cDNA libraries in λZAPII have been constructed so as to produce single-stranded DNA for hybridization subtraction and the production of subtractive libraries.

Publications:

Schweinfest CW, Fujiwara S, Lau LF, Papas TS. C-myc can induce the expression of G_0/G_1 transition genes. Mol Cell Biol 1988;8:3080-7.

Watson DK, McWilliams MJ, Lapis P, Lautenberger JA, Schweinfest CW, Papas TS. Human and mouse ets-2 genes encode for highly-conserved proteins. Proc Natl Acad Sci USA 1988;85:7862-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05570-02 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Expression of HIV-1 and HTLV-I Structural Gene Proteins in Prokaryotic Vectors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	T. S. Papas	Chief	LMO	NCI
Others:	J. A. Lautenberger	Research Chemist	LMO	NCI
	F. Wong-Staal	Biologist	LTCB	NCI
	R. Ascione	Research Chemist	LMO	NCI
	D. R. Hodge	Special Volunteer	LMO	NCI
	C. Jorcyk	Biologist	LMO	NCI
	C. W. Schweinfest	Staff Fellow	LMO	NCI

COOPERATING UNITS (if any)

Program Resources, Inc., Frederick, MD (K. Samuel); Bionetics Research, Inc., Frederick, MD (B. Felber, G. Pavlakis)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

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INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.06	0.76	1.30

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The human immunodeficiency virus, type-1 (HIV-1) env gene components spanning the entire gp120 and gp41 regions were expressed at high levels, typically, as ~ 2-15% of the total E. coli proteins. Each of the polypeptides were characterized serologically by Western blot using a panel of HIV-1-positive test sera and normal human sera as controls. With one exception, all of the expressed env products were immunoreactive using human sera from HIV-1-infected individuals in double-blind assays. Recently, we produced miligram quantities of several env gene-encoded polypeptides that do not contain the CD4 homologous residues, as well as an amino terminal polypeptide derived from gp41 that is very immunogenic and very highly reactive with almost every sera tested, thus far. A number of these expressed polypeptides were used as antigens to successfully generate polyclonal and monoclonal antibodies that have been immunologically characterized. Using truncated HIV-1 gp120-encoded polypeptides, we have detected the presence of a shared epitope containing a common sequence (Asa, Asn, Lys, Thr) present in two other vector-expressed products; this epitope, we found, is also recognized by antibodies present in the sera derived from AIDS patients, as well as the serum obtained from Dr. D. Zagury, who experimentally self-inoculated himself using a recombinant vaccine-expressed HIV-1 envelope protein. Dr. Zagury is presently testing a number of our expressed env gene products for immunoreactivity using his own sera, as well as sera obtained in field trials in Western and Central Africa.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

T. S. Papas	Chief	LMO	NCI
J. A. Lautenberger	Research Chemist	LMO	NCI
F. Wong-Staal	Biologist	LTCB	NCI
R. Ascione	Research Chemist	LMO	NCI
D. R. Hodge	Special Volunteer	LMO	NCI
C. Jorcyk	Biologist	LMO	NCI
C. W. Schweinfest	Staff Fellow	LMO	NCI

Objectives:

The human retroviruses, human immunodeficiency virus (HIV-1) and human T-cell leukemia virus (HTLV-I), have been shown to be associated with the diseases, AIDS and adult T-cell leukemia (ATL), respectively. Recently, another human retrovirus, the human immunodeficiency virus, type 2 (HIV-2), has been shown to cause acquired immunodeficiency disease syndrome (AIDS) in certain parts of Western Africa. Thus, it is possible that other human diseases are caused by, as yet, unidentified variants of human retroviruses. Therefore, it is of interest to investigate these human retroviruses and their associated diseases, both of which have an inherent hazard of working with live virus or infected patient sera, as well as the limitation in the supply and production using live virus proteins. To circumvent these factors, we have expressed a number of recombinant retroviral gene products of HTLV-I and HIV-1 and -2 in prokaryotic vector systems. This system permits preparation of large amounts of specific human retroviral structural proteins. These expressed envelope proteins have been purified to near homogeneity, and are being analyzed for their seroreactivity using antibodies from patients infected with the retroviruses, HIV-1, HIV-2 and HTLV-I. Such expressed retroviral envelope gene-encoded products can be used for both diagnostic and reagent purposes.

Methods Employed:

1. Construction of Plasmids. DNA fragments were produced by digesting plasmid DNA with restriction enzymes and were resolved on agarose or polyacrylamide gels. Fragments were isolated from agarose gels by dissolving the gel in NaI and adsorbing the DNA to glass beads. After washing the glass beads, the fragment was eluted with low-salt buffer and re-isolated from acrylamide gel electroelution. Fragments are joined by the action of DNA ligase and the resultant constructs were introduced in E. coli cells that had been made competent by CaCl treatment.

2. Protein Expression. Escherichia coli TAP 60 lysogenic strain or WPS-18 cells containing the vector plasmid, pJL6, or its modified derivatives, were induced at 42° for 1 hr. or less; proteins were extracted as described by Shoner et al. (Biotechnology 1985;3:151-4). Western blot analysis was performed as described (Science 1984;226:1094-7) on each of the fractions using an antibody raised against a peptide coded for by the retroviral gene, or with HIV-infected patient sera, where applicable. The major bands of reactivity that corresponded to the proteins in question were further purified

by isolation and successive washing of the bacterial inclusion bodies; the fractions solubilized in 8M urea or 7M guanidine-HCl. Often these proteins are visible on stained SDS-gels, and these particulate inclusion fractions are approximately 50% pure without any further chromatographic procedures.

3. The cells used contained proviruses derived from the human T-lymphotropic virus, type-I (HTLV-I)-infected T-cell line, C10/MJ, and the uninfected T-cell line, H9, or the acutely HIV-infected H9 cells. All bacterial cells were grown at the permissive temperature in LB or NZYDT broth. Proviral HIV clones, BH-8 and BH-10, were derived from unintegrated linear DNA obtained from acutely-HIV-infected H9 cells.

4. Computer Analysis of DNA and Protein Sequences. A wide variety of sequence analyses have been performed using the NCI-FCRF computer systems in connection with these studies (described in Major Findings). The extensive use of the University of Wisconsin software package implemented on the VAX computer of the Advanced Scientific Computer Laboratory (ASCL), Program Resources, Inc. for such applications as data entry, restriction mapping, protein sequence prediction, and alignment of multiple nucleic acids and protein sequences. Other analyses required the data base search procedures; GenBank SEQFT and others were run on the Cray-XMP supercomputer of the ASCL. Several specialized analytical appli-cations required the development of new programs. These included the development of a reverse translation program needed for the design of several deoxyinosine-containing oligonucleotide probes, as well as a program for the creation of hard-copy graphic display of these multiple alignments using laser printers that emulate Tektronics 4014 terminals.

Major Findings:

Nine bacterially-produced envelope gene-encoded protein fragments from different subregions of human immunodeficiency virus (HIV) glycoprotein molecules (gp120/gp41) have been characterized by Western immunoblot analysis using well-characterized HIV-positive human antisera and polyclonal antisera in double-blind assays. Monoclonal and polyclonal antibodies prepared against highly-purified gp120 and gp41 glycoproteins have been obtained. Each of the recombinant proteins, accounting for over 98% of the entire gp160 envelope glycoprotein of HIV, have been shown to be immunogenic and antigenic, with one exception being the product spanning the transmembrane moiety of gp41 env gene-encoded polypeptide.

The entire gp120 and gp41 envelope of HIV-1 strain, HTLV-IIIB, was expressed at relatively high, but variable levels (ranging from 1-15% of total E. coli proteins). These recombinant env-encoded polypeptides range in size from ~15KD to 40KD. Each polypeptide was first enriched by partial purification by extraction with detergents and chaotropic agents, and then characterized serologically using immunoelectroblotting methods in conjunction with a panel of HIV-1-positive sera and normal human (control) sera (i.e., sera that are negative for HIV-1 antibodies).

The origins and amino acid coordinates of each recombinant env polypeptide are: (1) 486 (gp120, 49-218); (2) 569 (gp120, 218-400); (3) 318 (gp120, 294-400); (4) 1061 (gp120/gp41, 294-647); (5) 719 (gp120/gp41, 405-647); (6) 347 (gp120/gp41); (7) 405-523, (8) 566 (gp41, 548-736); (9) 331 (gp41, 647-758); and (10) 503 (gp41, 732-863). With the exception of polypeptide 318, which forms part of the gp41 region spanning the membrane; all of the other env polypeptides are reactive on immunoblots. The patterns and levels of immunoreactivity were variable. Polypeptide 486 (N-terminal gp120), previously thought to be non-immunoreactive, was recently purified by us and shown to be quite immunoreactive in Western blot assays.

For analytical purposes and for use in antibody development, we purified the env polypeptides, 318 and 566. (a) Analytical preparation: Induced bacterial cell pellets from 2-liter cultures were extracted with detergent chaotrope buffers, and the enriched proteins (10-15 mg) solubilized with chaotropic solutions. Each polypeptide was purified by gel filtration, and by reverse-phase HPLC. The purified homogenous 318 (17KD) and 566 (22KD) env polypeptides were used as antigens in generating rabbit and mouse polyclonal and monoclonal antibodies, respectively. The purified comparative, diagnostic proteins were also used as antigens in ELISAs. (b) Scale preparation: Cultures of 350 liters of induced bacterial cells have yielded over 25 mg of env-specific product from 500-700 grams of E. coli. The polypeptides were extracted with detergent and chaotropic agents, as above, but the final crude protein fraction was washed more extensively and solubilized using ionic detergents, and then sized fractionated on a BioGel P60 column. Peak recombinant env protein fractions were rechromatographed and the purity of the env polypeptides was evaluated by 15% SDS-PA gels and by Western blot assay using monoclonal antibodies and human sera. Homogenous isolation of the purified env polypeptides was achieved by preparative electrophoresis on 15% polyacrylamide gels. The 318 (17KD), 569 (23KD), and 566 (22KD) recombinant HIV-1 env polypeptides have been purified using this large-scale protocol. These env polypeptides are currently being used in collaboration with Drs. Robert Gallo and Daniel Zagury for development as potential vaccines against the HIV-1 agent.

The antigenic properties of purified 566 and 318 env polypeptides have been utilized in comparative ELISA tests. These recombinant proteins were also compared to the authentic HIV-1 gp41 and a synthetic gp41 peptide by ELISA for their potential as sensitive and highly-specific HIV-1 diagnostic reagents. The 566 polypeptide was shown to be as sensitive (even at dilutions up to 1:300,000) and as specific as the authentic viral gp41 glycoprotein. These reactivities far exceed that of the 318 recombinant expressed polypeptide and synthetic oligopeptide for detecting HIV-1-positive sera.

The homogenous preparations of the recombinant env polypeptides of gp120 (318, 569, and 347) and gp41 (566 and 503) have been used to inoculate rabbits and mice to produce specific anti-envelope antibodies. The rabbit antiserum against the 17KD 318 polypeptide immunoprecipitates the authentic gp120 from H9-infected HTLV-IIIB cells; it also recognizes both gp120 and the 318 polypeptide in Western blot assays. Several mouse monoclonal antibodies (MAbs) against the 17KD 318, 23KD 569, and 22KD 566 env polypeptides

have been generated and used to screen the respective recombinant polypeptides. Significantly, some of the 569 MAbs can specifically immunoprecipitate authentic viral gp120 from H9 cells infected with either HTLV-IIIB or RF HIV-1 isolates. Antibodies to the 347 and 503 expressed polypeptides are currently being characterized, while the 486 antibodies are in preparation.

To aid in the identification and mapping of important immunogenic epitopes for human serum antibodies and mouse MAbs, we have begun the production of recombinant env polypeptides that are C-terminal truncations of our already existing bacterially-expressed HIV-1 env gene products. One clone, 569Δ, expressed sixfold greater levels of protein (~9.5KD). The use of this truncated 569 polypeptide in Western blot assays against 569 and 318 MAbs revealed the presence of a shared epitope containing the common sequence, Asn Asn Lys Thr, that is present in both the 318 and 569Δ polypeptide residues. Moreover, this sequence is apparently recognized by antibodies in sera of AIDS patients, as well as sera from an individual (D. Zagury) experimentally inoculated with a vaccine-recombinant HIV-1 envelope gene. Further studies are now in progress to map this common sequence and determine its biological significance as a potential immunogenic determinant.

We have also used partial protease treatment of purified 566 polypeptides to generate unique individual oligopeptides that were resolved by SDS-PAGE. These peptides were reacted on Western blots with either a) HIV-1-positive human sera; b) mouse monoclonal antibodies to intact 566 polypeptide or authentic viral gp41; or c) two serum samples obtained from Dr. D. Zagury of France (an experimentally-inoculated human). Immunoanalysis reveals that antibodies in naturally-infected HIV-1 sera recognized different epitope regions of the oligopeptides than did the mouse monoclonal antibodies. By contrast, antibodies from Dr. Zagury's serum did not react with any of the oligopeptides. However, the antibodies in Dr. Zagury's serum strongly recognized intact 566 recombinant expressed polypeptides, as well as the authentic viral gp41 glycoprotein. These results strongly suggest that naturally-infected humans and mouse antibodies against gp41 see different antigenic epitopes. This epitope may, yet again, be different from that seen by antibodies obtained from an experimentally-inoculated individual using an intact recombinant gp160 protein. We are currently attempting to purify and sequence these gp41 determinants.

HIV-2 envelope-specific polypeptides were expressed at levels of about 5-8% of total E. coli proteins; a number of recombinant polypeptides were obtained spanning the gp120 gp35 env gene-encoded sequences. They include: 1) 22KD of clone pMZ933 (amino acids 1-220), 2) 15KD of clone pMZ945 (aa214-345), 3) 23KD of clone pMZ922 (aa 333-536), 4) 20KD and 16KD species of clone pMZ921 (aa537-707) and 20KD of clone pMZ997 (aa 537-701), and 5) 18KD of clone pMZ1003 (aa 701-857).

Several gp35 and gp120 HIV-2 NIH-Z clones have been obtained that express relatively high levels of recombinant polypeptide products. Each recombinant env polypeptide was visible on Coomassie-stained 15% SDS-PA gels and was

further characterized serologically on Western blots against a panel of HIV-2-positive, HIV-1-positive, and normal human serum. The Western blot results showed that the most immunoreactive region of the HIV-2 envelope is the gp35 transmembrane protein. Two carboxyl-terminal HIV-2-specific gp35 antigens are currently being extensively purified for use in large-scale Western blot and ELISA screening tests, as well as for the development of HIV-2-specific mouse monoclonal antibodies.

In collaboration with Drs. Arthur Chen and Max Essex of Harvard, we have expressed a portion of the gp46 gene of the human retrovirus, HTLV-I, using the pJL6 and p806 E. coli vectors. In this study, we successfully expressed several HTLV-I gp46 containing env fusion polypeptides that are differentially immunoreactive on Western blots using HTLV-I-positive sera.

The results of our study with HTLV-I expressed polypeptides enabled us to conclude that the most immunoreactive (~93%) region of the gp61 glycoprotein resides in the C-terminal portion of gp46; the gp21 transmembrane envelope protein was less immunoreactive (~75%). Additionally, the recombinant env polypeptides that we produced, spanning the length of gp46, detect >99% of all HTLV-I-positive sera, suggesting their potential usefulness in the diagnosis of HTLV-I infections.

Publications:

DuBois GC, Samuel KP, Hanson CA, Zweig M, Showalter SD, Papas TS. Expression and purification of protein segments encoded by the envelope and 3'-orf genes of human immunodeficiency virus type 1. AIDS Res and Human Retroviruses 1988;4:419-32.

Lautenberger JA, DuBois GC, Kan NC, Samuel KP, Seth A, Papas TS. Expression of retroviral proteins in bacteria. In: Diwan A and Nakamura JM, eds. Proceedings of the Asia-Pacific conference on human retroviral infections. Honolulu: University of Hawaii Press (In Press)

Lautenberger JA, DuBois GC, Samuel KP, Seth AK, Showalter SD, Schweinfest CW, Zweig M, Papas TS. Expression of human retroviral proteins in a heterologous system. In: Groopman JE, Chen I, Essex M, Weiss R, eds. Early human retroviruses. UCLA Symposia on molecular and cellular biology, New Series, vol. 119. New York: Alan R Liss, Inc (In Press)

Papas TS, Samuel KP, Lautenberger JA, DuBois GC, Zweig M, Showalter SD, Seth A, Fisher RJ, Fujiwara S, Schweinfest C, Shih TY, Ascione R. Vector systems for the high-level expression of oncogenes, HTLV-I and HIV polypeptides. In: Albertini A, Paoletti R, and Reisfeld RA, eds. Molecular probes: technical and medical applications. New York: Raven Press, 1989;79-90.

Zweig M, Bladen SV, DuBois GC, Samuel KP, Showalter SD, Papas TS. Comparative analysis of gp41 antigens by enzyme-linked immunosorbent assays for detecting antibodies to human immunodeficiency virus type 1. AIDS Res and Human Retroviruses 1988;4:487-92.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05571-02 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies of E26 Avian v-ets and its Cellular Homologue in Mouse Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. Blair	Supv. Research Chemist	LMO	NCI
Others:	Q. Yuan	Visiting Fellow	LMO	NCI
	A. Seth	Visiting Scientist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI
	D. Watson	Research Microbiologist	LMO	NCI
	K. J. Dunn	Bio. Lab. Tech. (Micro)	LMO	NCI
	S. Ruscetti	Microbiologist	DCBD	NCI

COOPERATING UNITS *(if any)*
Nucleic Acid & Protein Synthesis Laboratory, PRI, Frederick, MD (M. Zweig, S. D. Showalter)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Microbiology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.9	1.4	0.5

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A replication-defective murine retrovirus, ME26, was previously constructed by inserting the avian gag-myb-ets sequences derived from the cloned avian acute leukemia virus, E26, into an Abelson murine leukemia virus (MuLV)-derived retroviral vector. NIH 3T3 cells infected with ME26 exhibit morphological alterations and increased proliferation. ME26 induces an increased incidence of leukemia, primarily erythroid and myeloid, when injected into newborn mice. Recently, we have constructed a recombinant provirus containing only the ets-specific sequences (v-ets). Cells transfected with this construct expressed the expected 60Kd v-ets protein and formed foci in the defined media containing 0.1% calf serum with 10- to 20-fold lower efficiency than those expressing the intact ME26 construct. Removal of 34 amino acids of p15-gag from ME26 or the v-ets viral constructs neither increased nor decreased their focus-forming activity, suggesting that the myristilation signal of murine p15 has no effect on viral activity in tissue culture. Comparison of biological activity of transfected v-ets in tissue culture with that of transfected chicken c-ets-1 suggests that 5′ ets sequences represent a critical region for biological function of ets in NIH 3T3 cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. Blair	Supv. Research Chemist	LMO NCI
Q. Yuan	Visiting Fellow	LMO NCI
A. Seth	Visiting Scientist	LMO NCI
T. S. Papas	Chief	LMO NCI
D. Watson	Research Microbiologist	LMO NCI
K. J. Dunn	Bio. Lab. Tech. (Micro)	LMO NCI
S. Ruscetti	Microbiologist	DCBD NCI

Objectives:

To study the mechanism and cooperative role of the myb and ets oncogenes of the avian erythroleukemia virus, E26, in oncogenesis.

To determine the biological function of v-ets and its cellular homologue in altering cell growth and hematopoietic development, and to develop biological assays to characterize these functions.

Methods Employed:

Standard methods of tissue culture of transformed and normal cells, DNA and RNA isolation from tumor tissues and tissue culture, Southern and Northern blotting using radioactive probes prepared from cloned DNAs, calcium-phosphate DNA transfection, measurement of tumorigenicity of cell lines in nude and other strains of mice, immunoprecipitation and protein gel analysis to detect specific cellular proteins.

Major Findings:

1. A provirus construct expressing only the v-ets sequence has reduced biological activity in comparison to the intact gag-myb-ets sequences of ME26. We have previously shown that NIH 3T3 cells infected or transfected by ME26, a murine retrovirus construct derived from avian E26 virus, form foci of mitogenically-stimulated proliferating cells. Foci of growing morphologically-altered cells are detected in defined media at low serum levels. Recently, we have constructed a recombinant provirus containing only the ets-specific sequences (v-ets). Cells transfected with this construct expressed the expected 60Kd v-ets protein detectable by monoclonal anti-ets antibodies. Proviral v-ets transfected NIH 3T3 cells form foci in defined media (QBSF) containing 0.1% calf serum, but with 10- to 20-fold less efficiency than those expressing the intact ME26 construct. We have previously proved that deletion of the avian gag and the 5′ third of myb from ME26 did not affect its biological activity, but that expression of the intact v-ets region is required. Taken together, these results suggest that structural or functional elements contained in the 3′ portion of the transduced myb sequences are required for mitogenic stimulation of mouse cells by ME26.

2. Presence of the myristilation signal of murine p15 has no effect on tissue culture activity of either ME26 or the v-ets viral constructs. ME26 was constructed by fusing the avian E26 gag-myb-ets coding region to the first 34 amino acids of the p15 gag of ets Abelson MuLV, in which the myristilated N-terminal glycine is required for the interaction with plasma membrane. In order to study the possible function of this myristilation signal of the ME26 in tissue culture, we modified the vector by removing 56 nucleotides of 3′ leader and the 34 amino acids of p15-gag. NIH 3T3 cells transfected by either ME26 or the v-ets viral constructs with removed p15-gag^{m} expressed slightly smaller proteins, but neither increased nor decreased their focus-formation activity in tissue culture.

3. 5′ ets sequences could be the critical region in biological function of ets. The major transcript of the proto-oncogene homologue of the ME26 ets sequences, chicken c-ets-1, differs from the transduced v-ets sequences at both the amino and carboxyl termini. Transfection experiments with the chicken c-ets-1 gene and v-ets, both under the vector containing mouse metallothionein promoter, has shown that the two constructs exhibit different mitogenic stimulating activities. Since substitution of 3′ c-ets-1 coding sequences for v-ets sequences in ME26 has no effect on viral activity in vitro, this suggests that 5′ ets sequences may be the critical region for the ets function.

4. Different NIH 3T3 cell lines have different responses to ME26 infection. Several NIH 3T3 cell lines were identical by fingerprint analysis and showed the same response to MSV infection in either DCF media or defined media contain low serum. However, these lines have significantly different responses to ME26 injection. In one case, detectable foci form only in defined QBSF media with a low percentage (<1%) of calf serum, while another forms foci in Dulbecco's MEM media containing a minimum of 2-3% serum. The addition of serum to injected cells maintained initially in low serum leads to foci appearing within 5-10 days. These results support the hypothesis that ME26 virus function in 3T3 fibroblasts may be related to serum factors and their ability to interact with specific cell factors in culture.

Publications:

Yuan CC, Kan N, Dunn KJ, Papas TS, Blair DG. Properties of a murine retroviral recombinant of avian acute leukemia virus E26: a murine fibroblast assay for v-ets function. J Virol 1989;63:205-15.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05572-02 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Isolation of Potential Oncogenes from Teleost Tumors

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. K. Watson	Research Microbiologist	LMO	NCI
Others:	R. J. Van Beneden	Guest Researcher	LMO	NCI
	D. G. Blair	Supv. Research Chemist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*
U.S. Army Biomedical Research and Development Laboratory, Ft. Detrick, Frederick, MD (W. Van der Schalie, H. Gardner)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.29	1.29	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Previous studies have suggested that fish have oncogene sequences homologous to those found in mammalian and avian species. We were the first to confirm the presence of fish oncogenes by isolating and sequencing the c-myc gene from rainbow trout (Van Beneden et al., 1986). In order to examine the role of fish oncogenes in fish tumors, we developed a transfection system in which fish DNA in the presence of calcium phosphate was transfected into NIH 3T3 cells. DNA was extracted from (1) lymphosarcomas from feral Northern pike (Esox lucius), (2) hepatocellular carcinomas and adenomas from feral white perch (Morone americana), and (3) chemically-induced hepatic tumors in Japanese medaka (Oryzias latipes). The transforming ability of fish tumor DNA was examined by standard focus assay, nude mouse assay, and colony selection assay. DNA from diethylnitrosamine-induced cholangiocarcinoma in medaka was the most efficient in transformation of NIH 3T3 cells. Tertiary transfectants caused fromation of tumors in nude mice of >20mm in one week following injection. Southern blot analysis of these transfectant DNAs hybridized to a medaka genomic DNA probe showed bands present only in tumor-induced transfectants. No bands were present in DNA from NIH 3T3 controls and cells transfected with non-tumorigenic medaka DNA. This suggests the presence of specific fish sequences in transformants which do not appear to be homologous to K-ras, H-ras, c-myc, M-met or v-erbB. Parallel secondary transfection experiments were done without additional pSV2neo sequences. DNA isolated from these transformed cells hybridized to pSV2neo sequences on Southern blots, while control DNA was negative. This suggests that the fish transforming gene and the plasmid DNA may have been closely integrated into the host genome. A library was prepared from Sau3AI partial digests of this DNA ligated into a lambda DASH vector. This library is currently being screened for pSV2neo-positive clones, which will then be used to "walk" to find the fish-specific sequences.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. K. Watson	Research Microbiologist	LMO	NCI
R. J. Van Beneden	Guest Researcher	LMO	NCI
D. G. Blair	Supv. Research Chemist	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

The purpose of this investigation is to examine DNA isolated from teleost tumors for the presence of transforming genes. These gene(s), isolated by transfection assay, will be cloned and characterized. This will allow us to determine whether they are cellular homologs of known oncogenes or if they represent a new oncogene sequence. Transforming genes isolated from chemically-induced lesions will also enable us to examine the mechanism of chemical carcinogenesis.

Methods Employed:

1. Tumors and normal tissue were isolated from both feral and laboratory-raised fish. Tissue was either used immediately for DNA isolation or frozen in liquid nitrogen and stored at -70° until used. White perch (Morone americana) were caught by trawling in the Back River (Chesapeake Bay). Tumors were characterized by histopathological analysis of a section of the liver. Northern pike (Esox lucius) were trapped at Ostego Lake, Michigan. Sections from the lymphosarcoma (external lesion) and internal organs were preserved for histopathological examination. Tumors (primarily hepatocellular carcinoma) were induced in laboratory-reared Japanese medaka (Oryzias latipes) by treatment with either (1) diethylnitrosamine (DEN), 200 mg/l, 48 hr exposure to 14-day old post-hatch embryos; or (2) methylazoxymethanol acetate (MAM-Ac), 50 mg/l, 4 hour exposure to 8-day-old unhatched embryos.

2. High molecular weight DNA was prepared by the quick-dounce homogenization method of C. Cooper. Individual tissues were homogenized in buffer containing sodium dodecyl sulfate (SDS) and proteinase K followed by phenol-chloroform extractions. When very cartilagenous tissue was used, it was pulverized in liquid nitrogen prior to homogenization. DNA was then precipitated in ethanol, resolubilized and incubated sequentially with RNase A and proteinase K. Following phenol-chloroform extraction and resolubilization, DNA concentration was estimated by absorbance at 260 nm.

3. In order to identify transforming sequences, high molecular weight DNA isolated from fish tumors and from normal tissues (control) was examined by transfection assay. A stock of NIH 3T3 cells (490 N3T) was obtained from Dr. Donald Blair (See Project #Z01CP05295-08). Cells were maintained at levels below confluency in Dulbecco-modified Eagle's medium supplemented with 10% fetal calf serum. To each plate of 3 x 10^5 NIH 3T3 cells, 25 μg of fish genomic DNA was co-transfected with 2 μg of a neomycin-resistant plasmid (pSV2neo) in the presence of calcium phosphate (Pellicer et al., 1980). A

total of four plates (100 μg fish DNA) of each sample was tested, which was expected to provide one genomic equivalent of DNA. Cells were grown in the presence of gentimycin (G418) for two weeks. Drug-resistant colonies were selected, harvested by trypsinization, and replated (in the absence of G418) in a standard focus assay, in a colony selection assay, and/or injected into athymic mice.

4. Standard focus assay. G418-selected cells were replated in media without G418 and grown to confluency. Plates were examined for foci. Foci were counted, picked and expanded. DNA was isolated by the Hirt extraction procedure.

5. Colony-selection assay. Cells from the same pool as above were replated in minimal media (QBSF or QBSF supplemented with 0.1% serum, Quality Biological). Cells that formed colonies were counted and DNA isolated, as above.

6. Nude-mouse assay. The remaining G418-selected cells were injected into athymic mice (1.5×10^6 cells/mouse). Mice were examined for tumors at the site of injection (positive results usually occurred $\leq$ 6-8 weeks). Tumors were excised and portions frozen for DNA extraction (see 2.) and/or diced and placed in media for growth of tumor explants.

7. Southern blots were prepared using DNA isolated from transfected cells, nude mouse tumors and tumor explants. These were hybridized to either known oncogene probes (under low stringency conditions) in order to identify homologous oncogenes or to high molecular weight fish probes (under high stringency conditions) to identify fish-specific sequences. Fragments used as hybridization probes included v-erbB, H-ras, v-myb, v-abl, v-erbA, v-sis, v-src (all from Oncor Science), c-myc (rainbow trout), v-ets (fragment E1.28, Watson et al., 1985), neu (from M. Barbacid), m-met (T. Kimetic).

8. In order to determine the ability of cells to respond to contact inhibition, cells from foci were examined for their ability to grow in soft agar (0.23%).

9. In order to test the efficiency of the transfections, parallel secondary transfections were performed using DNA isolated from foci from the primary cotransfection, but without the addition of more pSV_2neo. Foci were picked and expanded and DNA isolated, as described previously. DNA digests from these foci were run on Southern blots and hybridized to radiolabelled pSV_2neo. Tertiary transfections were also done using DNA from these secondary foci, again without the addition of more pSV_2neo plasmid.

10. A library was prepared using Sau3A1 partial digests of DNA from the tertiary transfectants (without the additional pSV_2neo plasmid) ligated into the lambda DASH vector. This library is currently being screened for pSV_2neo sequences.

11. Northern pike were collected at Ostego Lake, Gaylord, MI. Lymphosarcoma tumors were excised, a portion preserved in 10% formalin for histopathological confirmation of the neoplasm, the majority frozen for DNA/RNA extractions, and the remainder used to begin primary cell cultures. Cell cultures were initiated by either mincing tissue in sterile phosphate-buffered saline or trypsinizing tissues overnight at 4°C. Dispersed cells were placed in droplets on tissue culture dishes and allowed to adhere for 1-2 hours. Media (McCoy's Modified 5A, GIBCO) was added and cells incubated at either 4°C or room temperature (~20°C).

Major Findings:

1. DNA isolated from a diethylnitrosamine-induced cholangiocarcinoma in medaka very rapidly transforms NIH 3T3 cells in primary, secondary and tertiary transfections, as determined by standard focus assay, colony selection in minimal media (QBSF), and the nude mouse assay. Tumors in nude mice grow to 20 mm after only 1-1/2 weeks. Cells isolated from foci demonstrate anchorage-independence by growth in soft agar.

2. DNAs isolated from northern pike lymphosarcoma and white perch cholangioma is also able to transform NIH 3T3 cells, but is not as aggressive as the DNA isolated from medaka cholangiocarcinoma.

3. DNA isolated from a MAM-Ac-induced rhabdomyosarcoma had only a marginal ability to transform NIH 3T3 cells, although this is a very aggressive lesion. The findings suggest that perhaps a "transforming gene," if present, does not function well in this system.

4. Hybridization of Southern blots of EcoRI and BamHI digests of DNA isolated from secondary and tertiary transfectants (induced by medaka cholangiocarcinoma DNA) to medaka genomic sequences at high stringency reveal specific bands. These bands are not present in DNA isolated from NIH 3T3 cells alone or in DNA isolated from cells transfected by nontumorigenic medaka DNA. This suggests that the transformation of the NIH 3T3 cells is due to specific fish sequences. These sequences have not yet been identified, but don't appear to be homologous to H-ras, N-ras, K-ras, v-erbB, myc, neu or m-met, as determined by Southern blot hybridization.

5. The efficiency of transformation, as determined by secondary and tertiary transfections without additional pSV_2neo plasmid, was very good. Approximately 40 foci/μg DNA and 10-12 foci/μg DNA were observed in secondary and tertiary transfections, respectively. Southern blots of DNA digests from foci cells, when hybridized to pSV_2neo at high stringency, revealed homologous sequences present in transformed cells that were not present in control cells. These results suggest that pSV_2neo, or some portion of it, had integrated into the host cell genome at a site near that of the transforming gene. This will

allow us to use the plasmid as a marker to identify the fish transforming gene. A library has been prepared from Sau3AI partial digests of DNA from foci of a tertiary transfectant ligated into a lambda dash vector. This library is now being screened, using pSV_2neo as a probe. Once a positive clone is isolated, it can be used to walk in both directions in order to locate the fish transforming sequence.

6. Ten primary cell lines derived from northern pike lymphosarcoma are currently in culture. All 10 are growing in suspended culture and appear to be lymphocyte-related. Five colonies of attached cells grew out for two weeks in culture and then died. RNA is currently being isolated from original tumor tissue to look at oncogene expression.

Publications:

Van Beneden RJ, Henderson KW, Gardner HS, Blair DG, van der Schalie WH, Papas TS. New models for oncogene isolation in the study of carcinogenesis. In: Gardner HS, ed. Proceedings of the non-mammalian toxicity assessment research review, US Army Biomedical Research and Development (In Press)

Van Beneden RJ, Powers DA. Structural and functional differentiation of two clinally distributed glucosephosphate isomerase allelic isozymes from the teleost Fundulus heteroclitus. J Mol Biol Evol 1989;6:155-70.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05574-02 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of Drosophila melanogaster ets and ets-like Genes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	D. K. Watson	Research Microbiologist	LMO	NCI
Others:	L. J. Pribyl	IRTA Fellow	LMO	NCI
	R. Ascione	Research Chemist	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.34	0.80	0.54

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Organisms from humans to Drosophila have been found to contain cellular sequences and transcripts that are homologous to viral-onc genes. The normal function of the genes encoded for by these cellular sequences is unknown. Only by understanding the normal function of these cellular genes (c-onc) will we have a possible means to understand how they can become transforming upon transduction by the virus. With this goal in mind, this study using Drosophila was begun.

Cellular sequences homologous to the ets region of the chicken retrovirus, E26, have been found in Drosophila. The characterized portion of this gene corresponds to the last two exons of the chicken c-ets-1 gene and has over 90% homology to the chicken gene at the predicted amino acid level. This gene, designated D-ets, is located on chromosome 3R at position 58A/B and produces a single transcript of 4.7 Kb throughout development. Low stringency hybridization of Drosophila genomic DNA shows several other bands that also hybridize with a viral ets probe, E1.28. Hybridization of a cDNA library under these conditions led to the isolation of a cDNA clone which shows considerable homology to v-ets, but is not D-ets. This gene, called D-elg for Drosophila ets-like gene, has ~60% homology with D-ets and is located on chromosome 3R at 97D. It produces two transcripts of 2.3 and 2.0 Kb in embryo, pupae, and adult stages. A third gene, presently called E13B, was isolated by hybridization with v-ets. It is located on chromosome 3 at position 66A. This gene is expressed as a 1.6 Kb RNA species in only the pupal and adult stages. This Drosophila conserved the 3′ region of the ets gene in at least two different genes; providing a system that will determine the function of these genes. To study these functions a chimeric sequence (human 5′ end and Drosophila 3′ end) in a P-element vector is being constructed, which will be microinjected into embryos mutant flies. These experiments should supply important information on the developmental and biochemical function of the ets genes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. K. Watson	Research Microbiologist	LMO NCI
L. J. Pribyl	ITRA Fellow	LMO NCI
R. Ascione	Research Chemist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

To determine if there are sequences present in the Drosophila genome that are homologous to the v-ets sequences of the avian retrovirus, E26. If such sequences do exist, it will then be necessary to characterize this gene or gene family to understand the structural evolution of these genes. This would then allow one to develop a model for the domains along the ets protein which may have functional implications and may provide a functional assay.

Methods Employed:

1. Preparation of high molecular weight DNA from Drosophila collected during specific developmental stages and cells by treatment with proteinase K, followed by phenol-chloroform extraction. Vector DNA was prepared from phage and plasmid derivatives by phenol extraction and CsCl gradient centrifugation.

2. Digestion of genomic and clonal DNA with restriction enzymes and resolution of the resultant fragments on agarose and/or polyacrylamide gels. Nitrocellulose filters containing immobilized restriction fragments were prepared by the Southern blot technique and hybridized with specific ets probes. Specific DNA fragments were purified by electroelution or by extraction from low-melting agarose and were used either to prepare ets-specific DNA probes by nick-translation using E. coli DNA polymerase and DNase I or in the construction of a partial recombinant phage library by ligation of restriction fragments of eukaryotic DNA to λ vector DNA followed by production of phage by in vitro packaging.

3. Isolation of phage from the libraries containing virus-related sequences by hybridization of ets-specific probes to nitrocellulose filters containing phage DNA prepared from plaques by the procedure of Benton and Davis (Science 1977;196:180-2). Isolation of plasmid DNA's containing Drosophila cDNA's from colonies lifted from plates by the method of N. Brown (personal communication).

4. Subcloning of isolated Drosophila DNA fragments into appropriate plasmid vectors, as required.

5. DNA sequence analysis of cloned DNA by the method of Maxam and Gilbert (Methods Enzymol 1980;65:499-560). Also, dideoxy sequencing using the method of Sanger (Proc Natl Acad Sci USA 1977;74:5463-7) is employed.

6. Total cellular RNA from cultured cells or flies collected from defined developmental stages was prepared by the urea method (Biochemistry 1973;12: 2330-8). Separation of polyA+ and polyA- RNA by one cycle of purification through oligo (dT) cellulose. Characterization of RNA by electrophoresis on formaldehyde-agarose gels and Northern analysis.

7. Chromosomal in situ hybridization of Drosophila third instar larvae, using the method of Pardue and Gall (Methods Cell Biol 1975;10:1-16).

8. Total protein extractions from Schneider cells and developmental stages were done, with the resultant proteins electrophoresed on polyacrylamide gel and then transferred by electroblotting to nitrocellulose paper.

9. Western analysis using antibodies created against viral peptides were applied. The peroxidase conjugated antibody technique was used to detect reactivity.

10. P-element vectors, as designed by Rubin and Spradling (Science 1982;218:341-7 & 348-53), are used to carry into the Drosophila genome, a human Drosophila chimera. This chimera was produced by cloning the 5' portion of the human ets-2 cDNA with the 3' portion of the genomic Drosophila ets gene.

Major Findings:

1. In Drosophila there are three genes that are related to the v-onc gene, ets. These have been designated D-ets, D-elg, and E13B. The D-ets is a gene whose 3' end has been characterized from a single genomic clone. This region is highly homologous to the last two exons of the chicken c-ets-1 gene. It is also 94% homologous to the human ets-2 gene in this region. The 5' region of D-ets is now being isolated. A cDNA clone of 1.5 Kb from the D-elg gene has 50% homology to the D-ets gene. The E13B genomic clone is only partially characterized, but appears to be related to the 5' region of the v-ets gene. Partial cDNA's for the E13B gene, and possibly the D-ets gene, are now being analyzed.

2. D-ets is expressed as a 4.7 Kb transcript in embryo, larvae, pupae and adult stages. In contrast, Schneider cells, an embryonic cell line with a single point mutation in the D-ets coding region, encodes a 3.8 Kb transcript. D-elg is found to have two transcripts of 2.8 Kb and 2.0 Kb in embryo, pupae and adult stages with no expression in the larvae. The E13B gene is expressed only in the pupae and adult stages by a 2.6 Kb message.

3. All three genes have been localized to different chromosomes. D-ets is located on chromosome 2R at position 58A/B. D-elg is on the right arm of the third chromosome, at 97D, while E13B is on the left arm of the third chromosome, at position 66A.

4. The 3' end of the ets genes characterized to date are very highly conserved. This extensive homology persists in Drosophila, perhaps suggesting a conservation of function for a region with such great structural conservation.

5. The presence of a family of ets genes in Drosophila, as well as humans, has shed light on the evolution of a gene not only between species, but also within a species. This latter process might be extremely important for the production of different specialized genes.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05585-01 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Gene Expression in Colon Carcinoma and Polyposis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. W. Schweinfest	Staff Fellow	LMO NCI
Others:	T. S. Papas	Chief	LMO NCI

COOPERATING UNITS *(if any)*

Nucleic Acids & Protein Synthesis, Program Resources, Inc., Frederick, MD (K. Henderson); Hellenic Anticancer Institute, Athens, Greece (S. Kottaridis)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.54	0.54	0.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Colon cancer causes over 60,000 deaths in the United States each year and is second only to lung cancer as the cause of cancer deaths in the U.S. Colon carcinoma is largely unresponsive to chemotherapy and/or radiation; therefore, surgical resection is the treatment of choice. Nonetheless, over 60% of patients will die within five years of presentation. Patients who present with premalignant colonic polyps are considered to be at higher risk for developing colon cancer. Patients with an inherited predisposition for developing hundreds of polyps (called familial polyposis) are at nearly 100% risk of colon cancer by age 40. Finally, a large number of colon cancer originates from that mucosa (i.e., not from a polyp), especially in the proximal colon so flat a prehistory of polyposis which might lead to early detection is lacking.

Recently, restriction fragment length polymorphisms (RFLPs) loosely linked to familial polyposis have been described. Elevated ras gene expression is also observed in a large fraction of polyps and carcinomas. Finally, a pattern of chromosomal abnormalities is beginning to emerge. In order to directly investigate the major alterations in gene expression in colon carcinoma and in polyps compared to normal colonic mucosa, cDNA libraries from matched tumor/normal tissues were constructed. Subsequently, tumor minus normal and normal minus tumor subtractive cDNA libraries were prepared enriched for tumor-specific and normal-specific cDNA's, respectively. Two cDNA clones whose expression is greatly elevated in carcinoma tissue, but not in normal or polyp tissue, have been isolated. Other clones have been isolated and their expression levels will soon be characterized. Expression of these clones in colon carcinoma cell lines, as well as in other carcinomas (e.g., breast, ovary, endometrium) are being examined.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. W. Schweinfest	Staff Fellow	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

A key element in the fight to reduce the over 60,000 annual deaths in the U.S. due to colon cancer will be better and earlier detection of those individuals at risk. Presently, there are no good early markers. RFLP analysis used by some laboratories may be able to identify parts of chromosomes in which large alterations have occurred near relevant genes. The objective of this work is to identify specific genes whose expression is altered in polyps and in colon cancer relative to normal mucosa. These genes may prove to be markers for early detection of colon cancer and possibly other cancers.

Methods Employed:

1. RNA is isolated from matched tumor/normal tissues taken from patients with polyps or colon cancer. Where possible, three tissue samples are taken: tumor, proximal to tumor, and distal to tumor (normal).

2. cDNA libraries are constructed from tumor and normal tissue in a lambda vector capable of being induced to produce single-stranded phage bearing the cloned DNA in the plasmid vector bluescript.

3. ssDNA from the matched libraries are prepared; one of them is photobiotinylated, the libraries are hybridized, then the biotinylated DNAs (single-stranded and in hybrid) are removed on an avidin-agarose column. The non-biotinylated DNA is converted to double-stranded form and used to transform *E. coli*, resulting in a subtractive cDNA library enriched for tumor-specific (or conversely, normal-specific) sequences.

4. Subtractive library cDNA clones are used to probe Northern blots of RNA from the human tissues. This assay positively identifies clones preferentially expressed in tumor tissue.

5. Single-stranded subtractive DNA (from 3. above) is amplified using the polymerase chain reaction (PCR) method. It can then be used as a probe for doing differential hybridization to the starting libraries.

Major Findings:

1. Subtractive library formation seems capable of detecting transcripts which are highly expressed in their preferential tissue.

2. Two cDNA clones (Sπ5 and Sπ8) have been identified which are highly expressed in a colon cancer tumor relative to polyps or normal mucosa. Further characterization of these clones is underway.

3. PCR-amplified ssDNA enriched for tumor-specific or normal-specific sequences can be used as a probe to do differential hybridization to unsubtracted libraries. About two dozen clones isolated in this manner are being assayed by the Northern blot method.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05586-01 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Molecular Cloning of the Feline Immunodeficiency Virus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. W. Schweinfest	Staff Fellow	LMO NCI
Others:	T. S. Papas	Chief	LMO NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (K. Henderson); Cornell University, Ithaca, NY (F. Noronha)

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Laboratory of Molecular Oncology

SECTION
Carcinogenesis Regulation Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.24	0.24	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Feline immunodeficiency virus (FIV) produces a pathological condition in cats similar to AIDS in humans, produced by human immunodeficiency virus (HIV). Therefore, FIV is a useful animal model for studying AIDS. The Crandell feline kidney (CrFK) tissue culture cell line can be productively infected with FIV. Virions from infected CrFK cells were isolated, the genomic RNA purified and a cDNA library constructed. A series of overlapping clones, representing at least 6Kbp of the 9Kbp genome, have been isolated. FIV cDNA clones hybridize only to RNA and DNA from infected CrFK cells, thus ruling out that endogenous retroviruses have been induced. Partial sequence analysis of some clones resulted in a sequence which does not correspond to any retroviral sequences present in GenBank. This result suggests that, like its human counterpart, HIV, the feline immunodeficiency virus may contain additional open reading frames relevant to its pathology.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. W. Schweinfest	Staff Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

To clone and sequence the feline immunodeficiency virus. In vitro expression of FIV gene products and subsequent antibody production. These cloned DNAs and immunological reagents will be used to examine the effects of FIV infection on CrFK cells in vitro and on feline tissues in vivo.

Methods Employed:

1. cDNA cloning and DNA sequencing of clones.

2. Southern and Northern blot analysis of infected CrFK DNA and RNA, respectively.

Major Findings:

1. A set of overlapping cDNA clones comprising at least 6Kbp of the 9Kbp genome has been isolated.

2. Partial sequence analysis reveals DNA sequences with no homology to existing retroviral sequences now in GenBank. Therefore, FIV may contain unique genes analogous to the unique ORFs of HIV.

3. Infected CrFK cells contain integrated proviral DNA.

4. Infected CrFK cells express a 9Kbp transcript.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05587-01 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Search for ets-Related Sequences in Yeast DNA

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J. A. Lautenberger	Research Chemist	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Carcinogenesis Regulation Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

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0.19	0.19	0.0

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Members of the ets family of oncogenes have been been found in Drosophila, the sea urchin and several vertebrates. The Drosophila and sea urchin sequences correspond to the highly conserved region C of ets-1 or ets-2 of vertebrate ets. A series of oligonucleotides have been synthesized based on a consensus of all ets sequences. These oligonucleotides have been used as probes to detect possible ets-related sequences in the yeast, Saccharomyces cerevisiae. The identification of sequences that hybridize to several probes that are homologous to different regions of ets, but that are not related in sequence to each other, should allow the molecular cloning and characterization of yeast ets sequences.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

J. A. Lautenberger	Research Chemist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

While many animal ets genes have been cloned and sequenced, and some of the gene products have been identified, the function of ets has not yet been determined. The yeast, Saccharomyces cerevisiae, can be manipulated genetically in many ways that are not possible in other organisms. For example, if a yeast ets gene could be found, it could be used to determine the phenotype of yeast strains with either a totally non-functional ets gene or a yeast with a mutated ets gene. Studies of such strains could yield clues to the function of ets in yeasts and animals, including vertebrates.

Methods Employed:

1. Preparation of yeast DNA. Yeast DNA was prepared as described by Lautenberger and Chen (Gene Anal Techn 1987;4:87). Yeast cells were grown to stationary phase in YPD media, collected by centrifugation, washed and suspended in SZB buffer (SZB: 1 M sorbitol/0.1 M sodium citrate/0.06 M EDTA/0.6 mg/ml zymolyase-100T/0.8% 2-mercaptoethanol). After incubation for 40 min. at 37°, three ml of NDS (NDS: 0.01 M Tris-HCl/0.5M EDTA/1% sodium lauroyl sarcosinate (pH 9.2)/2 mg/ml protease K) were added and the solution was incubated for one hour at 50°. The reaction mixture was phenol and cloroform extracted and ethanol precipitated. The ethanol precipitate was suspended in 50 mM Tris-HCl/1 mM EDTA/0.2 mg/ml RNase A by gentle rocking overnight at 4°. The RNase was removed by chloroform extraction followed by ethanol precipitation and spooling on a glass rod. The final precipitate was dissolved in 50 mM Tris, pH 7.5/1 mM EDTA.

2. Southern hybridization. Yeast DNA was digested with restriction enzymes, and fragments were resolved on 1% agarose gels. After alkali denaturation and neutralization, the DNA was transferred to nitrocellulose filters by the method of Southern hybridization (J Mol Biol 1975;98:503). Oligonucleotide probes were synthesized based on segments of the ets sequences that are highly conserved at the amino acid level. Inosine was introduced at ambiguous locations. The oligonucleotides were radiolabeled by the activity of T4 polynucleotide kinase using high specific activity [^{32}P]ATP. Hybridizations were performed in 5XSSC/50 mM Tris-HCl, pH 7.5, 1 X Denhardt's solution/0.01 mg/ml E. coli tRNA/0.1% sodium pyrophosphate (1XSSC = 0.15M NaCl/0.015 M sodium citrate, pH 7.0; 1 X Denhardt's solution = 0.02% each BSA, polyvinylpyrrolidone, and Ficoll) at a temperature experimentally determined as optimal. Filters were washed 5 min. and 15 min. at room temperature and twice at hybridization temperature in 5XSSC/0.01% SDS.

Major Findings:

The hybridization patterns for several different probes have been determined. One set of common bands have been identified in digests from several different enzymes. These will be cloned and sequenced by making a phage lambda library from a size-fractioned digest.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05588-01 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Development of ets-2 Transgenic Mice

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Seth	Visiting Scientist	LMO NCI
Other:	D. Thompson	Biologist	LMO NCI
	T. S. Papas	Chief	LMO NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Transgenic Analysis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.48	0.18	0.30

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Transgenic mice offer a powerful model system for studying the molecular mechanisms for gene regulation during development. To study the role of ets proto-oncogenes in the normal developmental processes and tumorigenesis, we have generated transgenic mice that contain the ets-2 gene. The transgenic mice were generated by microinjection of ets-2 cDNA linked to the mouse metallothionein promoter into the pronuclei of one cell embryo. The injected embryos were implanted into the oviduct of pseudopregnant mothers and brought to term. One of the 22 pups was shown to contain the ets transgene by Southern blot analysis of DNA prepared from tails. The founder mouse (female) was bred again to produce offspring that contain the ets-2 transgene for further studies.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. Seth	Visiting Scientist	LMO NCI
D. Thompson	Biologist	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

To study the tissue-specific regulation and expression of ets genes in transgenic mice and their role in normal development and tumorigenesis.

Methods Employed:

1. Isolation of the ets-2 gene and analysis of high molecular weight DNA. The ets-2 gene linked to the mouse metallothionein promoter was isolated from the vector, pMME-18, by standard recombinant DNA techniques. Preparation of DNA from tails and Southern blot analysis have been previously published.

2. Production of transgenic mice. Microinjection of fertilized eggs with ets-2 DNA and implantation of fertilized eggs into the oviduct of foster mothers was done in collaboration with Bill Bullock at Stratagene.

Major Findings:

1. Production of ets-2 transgenic mice. We have generated a founder transgenic mouse that contains ets-2 sequences, linked to regulatory sequences, for efficient transcription from the mouse metallothionein promoter.

2. Breeding of ets-2 transgenic mice. The ets-2 transgenic mouse was bred to produce a large enough colony. So far, we have 15 ets-2 transgenic mice. These animals are under investigation for the tissue distribution and development of stage-specific expression in the ets-2 gene, as well as development of tumors or other physiological disorders.

Publications:

Gonzatti-Haces M, Seth A, Park M, Copeland R, Oroszlan S, Vande Woude GF. Characterization of the tpr-met oncogene p65 and the met proto-oncogene cell surface p140 tyrosine kinase. Proc Natl Acad Sci USA 1988;85:21-5.

Lautenberger JA, DuBois GC, Samuel KP, Seth A, Showalter SD, Schweinfest CW, Zweig M, Papas TS. Expression of human retroviral proteins in a heterologous system. In: Groopman JE, Chen I, Essex M, Weiss R, eds. Early human retroviruses, UCLA Symposia on Molecular and Cellular Biology, New Series, vol. 119. New York: Alan R Liss, Inc (In Press)

Papas TS, Samuel KP, Lautenberger JA, DuBois GC, Zweig M, Showalter SD, Seth A, Fisher RJ, Fujiwara S, Schweinfest C, Shih TY, Ascione R. Vector system for the high-level expression of oncogenes, HTLV-I, and HIV polypeptides. In: Albertini A, Paoletti R, Reisfeld RA, eds. Molecular probes: technical and medical applications. New York: Raven Press, 1989;79-90.

Psallidopoulos M, Seth A, DuBois G, Papas TS. Expression of the first exon open reading frame of human myc in E. coli as a fusion protein with v-mos oncogene product. Gene Anal Techn (In Press)

Seth A, Konopka A, Vande Woude GF. Localization of nucleic acid binding domain in mos. In: Brew K, Ahmad F, Bialy H, Black S, Fenna RE, Puett D, Scott WA, Van Brunt J, Voellmy RW, Whelan WJ, Woessner JF, eds. Advances in gene technology: protein engineering and production. Oxford/Washington DC: IRL Press, 1988;82.

Seth A, Thompson D, Chen SM, Papas TS. An inproved vector for the expression of proteins in all three translational reading frames. Gene Anal Techn (In Press)

Seth A, Vande Woude GF. The mos oncogene. In: Reddy EP, Skalka A, Curran T, eds. The oncogene handbook. New York: Elsevier, 1988;195-211.

Seth A, Watson DK, Blair DG, Papas TS. C-ets-2 proto-oncogene has mitogenic and oncogenic activity. Proc Natl Acad Sci USA (In Press)

Seth A, Watson DK, Blair DG, Thompson D, Dunn KJ, Papas TS. C-ets-2, a member of Down syndrome locus, has mitogenic and oncogenic activity. In: Rotundo RL, Ahmad F, Bialy H, Black S, Brew K, Chaitin MH, Glaser L, Magleby KL, Neary JT, Van Brunt J, Whelan WJ, eds. Advances in gene technology: molecular neurobiology and neuropharmacology. Oxford/Washington DC: IRL Press, 1989;155.

Seth A, Watson DK, Fujiwara S, Showalter SD, Papas TS. Expression of human and viral ets genes in E. coli production of human ets-2-specific monoclonal antibodies. Oncogene Res (In Press)

Wang LC, Gao C, Vaso WC, Seth A, Chen K. The role of v-mos in transformation, oncogenicity and metastatic potential of mink lung cells. Oncogene 1988;3: 267-73.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05589-01 LMO

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transformation of Primary Cells: Cooperation of ets with Other Oncogenes

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	A. Seth	Visiting Scientist	LMO	NCI
Others:	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*
Nucleic Acid and Protein Synthesis Laboratory, Program Resources, Inc.
Frederick, MD (S. Showalter)

LAB/BRANCH
Laboratory of Molecular Oncology

SECTION
Transgenic Analysis Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.18	0.18	0.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Carcinogenesis is a multistep process and involves several independent steps. At the cellular level, it has been suggested that at least two oncogenes are required to convert a normal cell into a tumor cell. The oncogenes that complement each other in transformation of primary cells have been classified into two groups. The first group contains nuclear oncogenes such as myc, p53, polyoma large T-antigen, jun, E1a of adenovirus, E7 of HPV; and the second contains cytoplasmic oncogenes such as ras and middle T-antigen of polyoma. The c-ets-2 gene product (p56ets) is localized in the nucleus, and it has been shown that the ets-2 gene is involved in cell proliferation. On the basis of nuclear localization and mitogenic activity, it has been suggested that the ets-2 is a member of the nuclear oncogene family (p53, myc, fos, myb). Recently, we have performed an extensive search in the NBRF protein data base for a secondary structure consisting of a negatively-charged short turn, followed by an α-helix, and found that this motif is also present in nuclear oncoproteins such as myc, ets-1, ets-2 and polyoma large T-antigen.

In NIH 3T3 cells we have recently shown that the ets-2 gene has mitogenic and transforming activity. To study the immortalization potential of the ets-2 gene and, also, whether it will complement the ras oncogene in transformation of primary cells, we have transfected rat embryo fibroblasts with c-ets-2 alone or ets-2 plus ras.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

A. Seth	Visiting Scientist	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

To study the immortalization potential of ets proto-oncogenes and its cooperativity with activated ras genes to transform rat embryo fibroblasts.

Methods Employed:

The procedures for restriction enzyme digestions, gel electrophoresis, isolation of DNA inserts from gels by electroelution, ligation of inserts to vector DNA, bacterial transformation, screening of clones by in situ colony hybridization, DNA probes by nick-translation, small-scale DNA preparation and analysis of the DNA by gel electrophoresis have been published.

The DNA transfection of NIH 3T3 cells by the calcium phosphate precipitation procedure, single cell cloning, growth in soft agar, growth in low-serum and serum-free medium, nude mice assay, high molecular DNA extraction, RNA extraction, Southern blots and Northern blots, and DNA and RNA blot hybridizations, were according to the published procedures.

Protein labeling with [^{35}S]-methionine, protein extraction, immunoprecipitation, and Western blotting with antibodies and [^{125}I] protein have been described previously.

Major Findings:

1. c-ets-1 and c-ets-2 retroviral vectors: The retroviral expression vectors containing the c-ets-1 and c-ets-2 proto-oncogenes were constructed by insertion of c-ets-1 or c-ets-2 cDNAs in a retroviral vector, fpgv-1.

2. c-ets-transfected cells contain multiple copies of ets genes: Rat embryo fibroblasts were transfected with ets-1, ets-2, myc and ras-containing vectors and the DNA prepared from cell lines were analyzed by Southern blot analysis. The cell lines carrying the integrated copies of transfected DNA (ets-1, ets-2, myc and ras) were further analyzed.

3. Establishment of rat embryo fibroblasts: Secondary rat embryo fibroblasts containing the integrated copies of the ets-2 gene have been in culture for more than 15 serial passages. In contrast, the control rat embryo fibroblasts are either growing very slowly or have already senesced. The immortalizing potential of ets-2 will be determined by the ability of transfected cells to grow indefinitely in culture (> 40-50 passages). The ability of the ets-2 gene to cooperate with activated ras genes in transformation of rat embryo fibroblasts is in progress.

Publications:

Gonzatti-Haces M, Seth A, Park M, Copeland R, Oroszlan S, Vande Woude GF. Characterization of the tpr-met oncogene p65 and the met proto-oncogene cell surface p140 tyrosine kinase. Proc Natl Acad Sci USA 1988;85:21-5.

Lautenberger JA, DuBois GC, Samuel KP, Seth A, Showalter SD, Schweinfest CW, Zweig M, Papas TS. Expression of human retroviral proteins in a heterologous system. In: Groopman JE, Chen I, Essex M, Weiss R, eds. Early human retroviruses, UCLA Symposia on Molecular and Cellular Biology, New Series, vol. 119. New York: Alan R Liss, Inc (In Press)

Papas TS, Samuel KP, Lautenberger JA, DuBois GC, Zweig M, Showalter SD, Seth A, Fisher RJ, Fujiwara S, Schweinfest C, Shih TY, Ascione R. Vector system for the high-level expression of oncogenes, HTLV-I, and HIV polypeptides. In: Albertini A, Paoletti R, Reisfeld RA, eds. Proceedings of the international symposium on molecular probes: technical and medical applications, New York: Raven Press, 1989;79-90.

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Seth A, Thompson D, Chen SM, Papas TS. An improved vector for the expression of proteins in all three translational reading frames. Gene Anal Techn (In Press)

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Seth A, Watson DK, Blair DG, Papas TS. C-ets-2 proto-oncogene has mitogenic and oncogenic activity. Proc Natl Acad Sci USA (In Press)

Seth A, Watson DK, Blair DG, Thompson D, Dunn KJ, Papas TS. C-ets-2, a member of Down syndrome locus, has mitogenic and oncogenic activity. In: Rotundo RL, Ahmad F, Bialy H, Black S, Brew K, Chaitin MH, Glaser L, Magleby KL, Neary JT, Van Brunt J, Whelan WJ, eds. Advances in gene technology: molecular neurobiology and neuropharmacology. Oxford/Washington DC: IRL Press, 1989;155.

Seth A, Watson DK, Fujiwara S, Showalter SD, Papas TS. Expression of human and viral ets genes in E. coli production of human ets-2-specific monoclonal antibodies. Oncogene Res (In Press)

Wang LC, Gao C, Vaso WC, Seth A, Chen K. The role of v-mos in transformation, oncogenicity and metastatic potential of mink lung cells. Oncogene 1988;3: 267-73.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05590-01 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of the Products of ets Gene Family

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. J. Fisher	Expert	LMO	NCI
Other:	S. Koizumi	Visiting Fellow	LMO	NCI
	S. Fujiwara	Visiting Associate	LMO	NCI
	A. Seth	Visiting Scientist	LMO	NCI
	L. Fleischman	ITRA Fellow	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS *(if any)*

Nucleic Acid and Protein Sythesis Laboratory; Program Resources, Inc., Frederick, MD (N. Bhat, S. Showalter); Centro de la Investigacions, Madrid, Spain, (S.M. Diaz de la Espina).

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Transgenic Analysis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.06	1.06	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The ets-gene family consists of three genes (ets-1, ets-2 and erg) homologous to the v-ets oncogene carried by the avian leukemia virus, E26. To characterize the products of the human ets-gene family, we have generated monoclonal antibodies (MAbs) specific for the proteins of the ets-gene family. Thus, MAbs to the ets-1 protein have been isolated and used to characterize the ets-1 protein in detail. The human ets-1 gene product consists of multiple proteins: four major proteins and two minor proteins. By using a combination of the MAbs and a set of polyclonal antibodies against different epitopes of the ets-1 protein, we found that two of four major proteins appeared to be missing exon 7, suggesting that these proteins were generated by alternative splicing of the mRNA. By subcellular fractionation and immunoelectron microscopy, the multiple ets-1 proteins were distributed both in the nucleus and cytoplasm. In vivo labeling with [32P]-orthophosphate showed that the proteins were heterogeneously phosphorylated; two minor proteins were demonstrated to be phosphorylated forms of the upper two major proteins, which themselves appeared to be phosphorylated to a certain extent, while the lower two major proteins, which appeared to delete exon 7, were not phosphorylated. Generation of the multiple proteins and the heterogeneity of their localization and phosphorylation may imply some functional significance for the human ets-1 gene product.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. J. Fisher	Expert	LMO	NCI
S. Koizumi	Visiting Fellow	LMO	NCI
S. Fujiwara	Visiting Associate	LMO	NCI
A. Seth	Visiting Scientist	LMO	NCI
L. Fleischman	IRTA Fellow	LMO	NCI
T. S. Papas	Chief	LMO	NCI

Objectives:

The major objective of this project is the development of highly-specific, immunological probes for the proteins encoded by the ets-gene family in order to characterize the biological and biochemical functions of these gene products. Monoclonal antibodies (MAbs) are particularly useful in many aspects: 1) MAbs against peptides derived from exons allow analysis of protein structure. Additionally, MAbs against functional domains (DNA binding, nucleotide binding, nuclear localization, and phosphorylation site, etc.) allow a functional characterization of the proteins. 2) MAbs allow the immunoaffinity purification of the ets proteins from cells. 3) MAbs against exposed epitopes of the ets proteins allow a) functional studies by microinjection of the MAbs into living cells; and b) immunohistochemical characterizations of the ets proteins in human tissue, such as thymus, brain, etc. Detailed analysis of the structure and function of the ets proteins should give insight to understand the molecular mechanisms by which these proteins mediate normal cell growth and differentiation, as well as malignant transformation.

Methods Employed:

The protein used for immunogen is prepared from ets-1, ets-2 and erg genes which have been expressed in E. coli, or from oligopeptides synthesized from the predicted amino acid sequences of these genes. Balb/c mice are immunized with these proteins and hybridomas are developed by standard procedures. Hybridoma supernatants are screened for specific monoclonal antibodies by enzyme-linked immunosorbent assay (ELISA) and further examined by immunoblotting, immunoprecipitation and immunofluorescence. Proteolytic fragment profiles of the detected proteins are obtained by digestion with Staphylococcal V8 protease, chymotrypsin, or trypsin, followed by one-dimensional or two-dimensional electrophoresis analysis. The ets proteins are purified by affinity chromatography using HPLC. N-terminal amino acid of the purified protein are sequenced by the protein sequencer. Microinjection is performed by using the microinjector.

Major Findings:

This year we have characterized the human ets-1 protein by using a specific monoclonal antibody (MAb) as a probe. This MAb recognized multiple proteins, four major (p51, p48, p42 and p39) and two minor (pp52 and pp49), by immunoprecipitation of the [^{35}S]-methionine-labeled CEM cell line.

These multiple proteins were all related by peptide mapping using Staphylococcal V8 protease. Furthermore, they were all recognized by the other ets-1-specific antibody, indicating that they were all ets-1 proteins. Combination of the MAb with a series of antibodies against different epitopes of the ets-1 protein showed that both p42 and p39 deleted a region in an exon represented by the genomic clone, pRD700 (chicken ets-1 exon 7 homologue), suggesting that these proteins were generated by alternative splicing of the particular exon. By subcellular fractionation and the immunoelectron microscopic technique, the ets-1 proteins were distributed both in the nucleus and cytoplasm; p52 and p51 were localized predominantly in the cytoplasm, p48 and p39 were mainly in the nucleus, and pp49 and p42 were in both compartments. In vivo labeling with [^{32}P] Orthophosphate showed the heterogeneous phosphorylation of the ets-1 proteins. pp52 and pp49 were demonstrated to be the phosphorylated forms of p51 and p48, which themselves appeared to be phosphorylated to a certain extent. In contrast, neither p42 nor p39 was phosphorylated. Generation of the multiple proteins by alternative splicing, as well as by post-translational modification and their heterogeneous localization and phosphorylation, may imply functional significance of the human ets-1 proteins.

Publications:

Bhat NK, Showalter SD, Fujiwara S, Fisher RJ, Koizumi S, Papas TS. Regulation of ets genes during cell proliferation and differentiation. In: Rotundo RL, Ahmad F, Bialy H, Black S, Brew K, Chaitin MH, Glaser L, Magleby KL, Neary JT, Van Brunt J, Whelan WJ, eds. Advances in gene technology: molecular neurobiology and neuropharmacology. Oxford/Washington, DC: IRL Press, 1989; 130.

Fujiwara S, Koizumi S, Fisher RJ, Bhat NK, Papas TS. Antibody to the T-cell receptor complex stimulates phosphorylation of the ets-2 protein. In: Rotundo RL, Ahmad F, Bialy H, Black S, Brew K, Chaitin MH, Glaser L, Magleby KL, Neary JT, Van Brunt J, Whelan WJ, eds. Advances in gene technology: molecular neurobiology and neuropharmacology. Oxford/Washington, DC: IRL Press, 1989; 132.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05591-01 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biological Characterization of the ets Proteins

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. J. Fisher	Expert	LMO NCI
Others:	L. F. Fleischman	IRTA Fellow	LMO NCI
	S. Fujiwara	Visiting Associate	LMO NCI
	S. Koizumi	Visiting Fellow	LMO NCI

COOPERATING UNITS (if any)

Nuc. Acid. & Prot. Syn. Lab., PRI, Frederick, MD (M. Zweig, S. Showalter, N. Bhat, G. DuBois)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Transgenic Analysis Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.05	1.05	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Several approaches have been initiated to investigate the cellular function of the ets gene products in T-cells and astrocytoma cells. Expression and phosphorylation of ets proteins during different stages of the cell cycle is being studied in CEM cells synchronized by centrifugal elutriation. Intracellular Ca-ion can be measured fluorometrically in studies designed to elucidate the role of ets in the Ca-ion mediated intracellular signaling pathways. The function(s) of ets protein in the astrocytoma cells are being analyzed using a variety of biochemical and cell biological techniques, including micro-injection. All of these studies utilize the antibodies and methods of ets protein isolation previously developed in this laboratory.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. J. Fisher	Expert	LMO	NCI
L. F. Fleischman	IRTA Fellow	LMO	NCI
S. Fujiwara	Visiting Associate	LMO	NCI
S. Koizumi	Visiting Fellow	LMO	NCI

Objectives:

The major objective of this project is to study the biological function of the proteins encoded by the c-ets-1 and c-ets-2 proto-oncogenes. Research will focus on the possible role of these proteins in cellular proliferation and signal transduction.

Methods Employed:

Biological Materials: The human T-cell leukemia line, CEM, which produces both c-ets-1 and c-ets-2, is being used in synchronization experiments. Recently, we have found expression of both c-ets-1 and c-ets-2 in the human astrocytoma line, 1321N1. Preliminary findings indicate that the pattern of ets-1 expression in 1321N1 cells differs from that seen in CEM cells, possibly due to alternative RNA splicing or processing. This will be a subject of continuing investigation. Normal tissues, such as mouse thymocytes and primary astrocyte cultures, will also be used to further explore the role of ets in these different cell lineages.

Cell cycle analysis: The pattern of ets-1 and ets-2 expression at different phases of the cell division cycle is being analyzed in CEM cells using the method of centrifugal elutriation. This technique exploits the principle of counterflow centrifugation to rapidly and gently sort cell populations according to cell size. Since volume increases during passage through the cell cycle, temporally-synchronized populations can be obtained in this manner, with minimal perturbation of cell metabolism. Staged cells are then analyzed by various methods, including metabolic labeling, immunoprecipitation and Western blotting using the ets-1 and ets-2-specific monoclonal antibodies recently developed in our laboratory. In this way, synthesis, phosphorylation state, half-life and steady-state levels of ets proteins at different stages of the cell cycle will be characterized.

Major Findings:

ets proteins and intracellular signals: Previous work has shown that ets proteins are phosphorylated in response to agents which increase intracellular Ca++. In addition, it has been found that activation of protein kinase C leads to a significant increase in the half-life of the ets-2 protein. Thus,

our current working hypothesis is that the ets proteins may function as intermediary signal transducers which respond to changes in Ca++ and other second messenger molecules in the cytoplasm, and also act in the nucleus to effect gene expression. We will utilize the Ca++-sensitive fluorescent probe, fura-2, to directly measure intracellular Ca++ levels and changes in Ca++ in response to various treatments in living cells. This should allow us to substantiate earlier findings and to assess, in much greater detail, the role of ets in the wide range of Ca++-mediated processes involved in cell growth and differentiation. The same instrumentation will also enable us to measure intracellular pH, K+ and other ions fluorometrically. This approach will also be applied to the synchronized cells described above, and will be used in studies of ets protein function in signal transduction in astrocytoma cells. The presence of high levels of ets-1 protein in both T-cells and astrocytes suggests it may function in some pathway important in both cell types, possibly in relation to growth stimulation by analogous signals. This could shed light on the interaction between the nervous and immune systems, as well as mechanisms of oncogenesis.

Microinjection studies: Biological function of ets will be probed using techniques for microinjection of single cells. Molecules of interest for microinjection include ets proteins produced by recombinant DNA techniques, monoclonal antibodies to ets proteins (likely to block ets function), and both sense and anti-sense ets mRNA. The astrocytoma cells are adherent and, therefore, particularly well suited for microinjection.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05592-01 LMO

PERIOD COVERED: October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Expression of HIV-2 env Gene Products in E. coli

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	T. S. Papas	Chief	LMO NCI
Others:	J. A. Lautenberger	Research Chemist	LMO NCI

COOPERATING UNITS (if any)

PRI, Frederick, MD (M. Zuber, K. Samuel, M. Zweig)

LAB/BRANCH: Laboratory of Molecular Oncology

SECTION: Carcinogenesis Regulation Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.21	0.21	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☑ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Human immunodeficiency virus, type-2 (HIV-2), the West African counterpart of the acquired immunodeficiency syndrome (AIDS) virus, is related to, but is quite distinct from HIV-1. The HIV-2 NIH-Z genome is approximately 9.4 kb long with an env gene of about 2.7 kb which codes for an envelope protein of 856 amino acids. The gp160 envelope protein is matured into a 120 Kd exterior glycoprotein (gp120) and a transmembrane protein of 35 Kd (gp35). The gp120 and gp35 envelope proteins of HIV-2 NIH-Z show about 32% and 39% homology, respectively, with the human T-lymphotropic virus, type-IIIB (HTLV-IIIB) strain of HIV-1. The chief objectives of this project are (1) to develop a diagnostic antigen for accurately detecting antibodies to HIV-2 in all infected human sera and (2) to use the bacterially-expressed HIV-2 env gene products in some vaccine applications. We have expressed the env gene of the NIH-Z isolate of HIV-2 as five overlapping fragments in E. coli. The env open reading frames (ORFs) were initially expressed as tripartite fusions in between the 12 amino-terminal codons of the lambda cII gene and the lacZ (β-galactosidase) gene of E. coli that are under the transcriptional control of the λ PL promoter of the expression plasmid pWS50. The β-galactosidase portion of the fusion protein was then deleted at the DNA level by generating in-frame translational stops or translational frame-shift mutations. One of the ORFs spanning the amino acid residues 536 through 705 of the HIV-2 env gene directed the synthesis of a 20 Kd protein that was serologically specific for identifying antibodies to HIV-2. This antigenic protein is produced at levels approximately 5% of the total cellular proteins. No significant immunological cross-reactivity was observed with HIV-1-positive sera or with normal control sera in immmunoblot assays with a crude preparation of this protein. Work is now in progress on the immunological characterization of the gene products of the remaining four HIV-2 env ORFs.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

T. S. Papas	Chief	LMO NCI
J. A. Lautenberger	Research Chemist	LMO NCI

Objectives:

(1) To develop a diagnostic antigen for accurately detecting antibodies to HIV-2 in all infected human sera, (2) to use the bacterially-expressed HIV-2 env gene products in some vaccine applications.

Methods Employed:

Plasmids, bacterial strain and HIV-2 clone: E. coli strain TAP56 (gift from Drs. D. Court and T. Patterson, BRI, NCI-FCRF, Frederick, MD) is a lambda lysogen that harbors a mutant temperature-sensitive repressor coded by phage λ cI_{857}. This strain is grown in Luray broth at a permissive temperature (32°C). A SacI subclone containing the entire env-nef-3′ LTR sequences of $HIV\text{-}2_{NIH\text{-}Z}$ provirus was kindly provided by Dr. Robert Gallo, Laboratory of Tumor Cell Biology, NCI. The bacterial expression vector, pWS50, and the plasmid, pWS60 (gifts from Dr. D. Court, BRI, NCI-FCRF, Frederick, MD), contain the well-regulated λ_{PL} promoter, N-terminal 13 amino acids of the λ cII gene with its ribosome bindings site (RBS) and ATG start codon, and the lacZ gene of E. coli.

DNA manipulations: Plasmid DNA preparations, restriction analysis of plasmid DNA, purification of DNA fragments from agarose gel slices, Bal31 treatment of purified DNA fragments, ligations and transformation using competent E. coli cells were done following standard recombinant DNA techniques.

Analysis of recombinant env proteins: Induction of recombinant plasmids for protein expression, partial purification of recombinant proteins, SDS-polyacrylamide gel electrophoresis and Western blot analysis were done following published procedures. Sera from HIV-2-infected individuals were kindly provided by Dr. Phyllis Kanki of Harvard Medical School.

Major Findings:

HIV-2 specific recombinant antigen: An open reading frame spanning the amino acid residues 536 through 705 of the HIV-2 env gene directed the synthesis of a 20 Kd protein in E. coli that was specific for identifying antibodies to HIV-2. This antigenic protein is produced at levels approximately 5% of total cellular proteins, and cross-reacted very strongly with eight different HIV-2-positive human sera, even at very high dilutions of 1:500. No significant immunological cross-reactivity was observed with HIV-1-positive or with normal control sera in immunoblot assays with a crude preparation of this protein.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05593-01 LMO

PERIOD COVERED: October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Transcriptional Regulation of the Human ETS-2 Oncogene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	D. K. Watson	Research Microbiologist	LMO	NCI
Others:	G. Mavrothalassitis	Visiting Fellow	LMO	NCI
	T. S. Papas	Chief	LMO	NCI

COOPERATING UNITS (if any)

LAB/BRANCH: Laboratory of Molecular Oncology

SECTION: Carcinogenesis Regulation Section

INSTITUTE AND LOCATION: NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.04	1.04	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Our understanding of gene regulation has been facilitated by structural and functional analysis of their promoter region. We have determined the nucleotide sequence of the human ETS-2 oncogene promoter region. The transcription initiation sites were determined by various mapping methods. The presence of multiple initiation sites is consistent with the absence of typical TATA and CAAT "boxes" in the surrounding sequence. Several repeat regions, two consensus AP2 and three putative Sp1 binding sites can be identified in the promoter region. A GC element with dyad symmetry is seen next to the major initiation site proximal to an unusually long (~ 250 bp) polypurine polypyrimidine tract. This polypurine polypyrimidine track is essential for the activity of the ETS-2 promoter. Fusions of a series of deletion fragments of the promoter with the CAT gene and subsequent transfection into human cell lines indicate that the sequences from -3600 bp to -159 bp are responsible for the promoter function. Further truncation gradually reduces the activity of the promoter. A number of S1 hypersensitive sites have been identified proximal to the transcription initiation region near the cis regulatory elements of the ETS-2 promoter. The polypurine polypyrimidine track, proximal to the promoter can act as a transcriptional activator in a transfection assay when it is placed upstream of the α-globin gene promoter. The identified ETS-2 promoter will be used to isolate and characterize trans factors involved in the ETS-2 gene transcriptional regulation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

D. K. Watson	Research Microbiologist	LMO NCI
G. Mavrothalassitis	Visiting Fellow	LMO NCI
T. S. Papas	Chief	LMO NCI

Objectives:

The purpose of this investigation is to identify the region from where the human ETS-2 oncogene is transcribed. The analysis of this region will allow us to understand its transcriptional regulation and its possible deregulation in human malignancies.

Methods Employed:

1. Preparation of high molecular weight DNA from eukaryotic cells and nuclei by treatment with proteinase K, followed by phenol-chloroform extraction.

2. Digestion of DNA with restriction enzymes and resolution of the resultant fragments on agarose and/or polyacrylamide gels. Specific DNA fragments were purified by electroelution or by extraction of low-melting agarose.

3. Preparation of DNA probes using purified onc-specific DNA by nick-translation using E. coli DNA polymerase (Klenow fragment).

4. Preparation of nitrocellulose filters containing immobilized restriction fragments by the Southern blot technique and hybridization of onc probes.

5. Subcloning of DNA fragments into appropriate plasmid vectors, as required.

6. Preparation of nested deletion with Exonuclease III and Mung Bean nuclease.

7. DNA sequence analysis of cloned DNA by the method of Sanger (Proc Natl Acad Sci USA 1977;75:5463-7).

8. Total cellular RNA from cultured cells or tissues was prepared by the guanidine isothiocyanate and LiCl/urea methods. Separation of polyA+ and polyA- RNA by purification through oligo(dt) cellulose. Characterization of RNA by electrophoresis on formaldehyde-agarose gels and Northern analysis.

9. Identification of promoter and regulatory sequences present in genomic clones of the cellular homologs of oncogenes. To include nucleotide sequence analysis and functional assays using expression of defined reported genes, such as chloramphenicol acetyltransferase (CAT), after transfection in eukaryotic cell lines.

10. Nuclease protection assays to define the 5′ end of transcripts. Verification by primer-extension of mRNA.

11. Identification of nuclease hypersensitive sites in chromosomal and supercoiled DNA by S1 nuclease digestion under appropriate conditions and subsequent agarose electrophoresis.

Major Findings:

1. The sequence surrounding the human ETS-2 gene transcription initiation site(s) was determined.

2. The mRNA transcription initiation sites were mapped by nuclease protection and primer extension analysis. Multiple initiation sites were detected spanning a region of about 100 bp. More than 80% of the ETS-2 mRNA starts within 10 bp.

3. The levels of initiation from the determined sites is consistent with the steady state mRNA levels among various cell lines tested.

4. All three human ETS-2 mRNAs start from this region, as determined by Northern analysis using specific 5′ probes.

5. The ETS-2 promoter sequence lacks typical TATA and CAAT "boxes" consistent with the multiple initiation sites. Putative AP2 and Sp1 binding sites are located within 300 bp 5′ from the major initiation sites.

6. An unusually long (~ 250 bp) polypurine polypyrimidine track is adjacent to the initiation sites. Several repeats exist in this region. One of these shares homolog with sequences found in the promoter of other genes.

7. A GC element with dyad symmetry next to the major initiation sites may be important for the positioning of the transcription initiation. Identical elements can be detected next to the initiation sites of other genes with similar promoter structure.

8. The region surrounding the major initiation sites is capable of driving the transcription of a report gene after transfection in human cell lines.

9. Deletion analysis of this region indicates that the sequence from -159bp to +140bp is necessary for the maximum activity of the promoter. Truncation of this region gradually decreases the promoter strength.

10. The ETS-2 promoter does not respond to TPA in transient transfection conditions, indicating that the determined increase in steady state levels of mRNA, is due to post-transcriptional events.

11. The -395 bp, -15 bp regions of the ETS-2 promoter can increase the transcription of α-globin gene promoter when placed in the correct orientation. Truncation of the 5′ end of this region reveals an initial

increase and the gradual decrease of the stimulatory effect. The pattern of the induction is parallel to the one produced from the deletion analysis of the ETS-2 promoter strength pattern.

12. Two CT-rich oligonucleotides from this region can induce the α-globin promoter in a dosage-dependent orientation independent manner. The effect is almost identical for both oligonucleotides, indicating that the polypurine polypyrimidine track of the ETS-2 promoter may act as a transcription activator.

13. The polypurine polypyrimidine track of the ETS-2 promoter is nuclease hypersensitive at the chromatin and supercoiled DNA levels. The nuclease hypersensitive sites appear to be in close relation with cis elements necessary for the ETS-2 gene transcription.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05594-01 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Identification of Potential Suppressor Genes for ras Transformation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	T. Y. Shih	Research Chemist	LMO	NCI
Others:	Y. Ogiso	Visiting Fellow	LMO	NCI
	L. S. Ulsh	Microbiologist	LMO	NCI
	L. Gutierrez	Visiting Fellow	LMO	NCI
	A. Seth	Visiting Scientist	LMO	NCI

COOPERATING UNITS (if any)

Osaka University, Osaka, Japan (H. Okayama); Massachusetts General Hospital, Boston, MA (B. Seed); Hokkaido University, Sapporo, Japan (N. Kuzumaki)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.12	0.82	0.30

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of the present project is to identify candidate tumor suppressor genes, which may eventually yield to analysis of human cancers. The strategy we used is to isolate morphologically-flat revertants from NIH 3T3 cells transformed by the EJ-ras oncogene, following transfection with cDNA expression libraries containing tumor suppressor genes from normal cells. The functional cDNA clones can then be recovered from the flat revertant cells and be further characterized. Using a methionine starvation procedure as a negative selection for flat revertants, we are able in a typical experiment, to isolate more than 40 morphologically flat clones from EJ-ras-transformed cells following transfection with a cDNA library constructed from human fibroblasts. Experiments are in progress to characterize these flat revertants and to recover the cDNA clones from them.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliation of Professional Personnel Engaged on this Project:

T. Y. Shih	Research Chemist	LMO NCI
Y. Ogiso	Visiting Fellow	LMO NCI
L. S. Ulsh	Microbiologist	LMO NCI
L. Gutierrez	Visiting Fellow	LMO NCI
A. Seth	Visiting Scientist	LMO NCI

Objectives:

Human carcinogenesis is a multistep and multifactor process that progressively converts normal cells into malignant cancerous states. Approximately 50 oncogenes that accelerate the development of cancer have been identified. It is also apparent that normal cells possess suppressor genes that retard tumor development. Somatic cell hybrids between malignant and normal cells very often display a normal phenotype. Genetic studies of human and animal cancers have identified genetic loci; loss of their function predisposes individuals to tumor development. One of these recessive tumor suppressor genes, the human retinoblastoma (Rb) susceptibility gene, has recently been molecularly cloned. We hypothesized that in order to prevent a normal cell from becoming malignant through inadvertent activation of various proto-oncogenes, each cell must also possess a group of tumor suppressor genes to restrain the normal cell from malignant conversion. Mutations that result in loss of these suppressor functions will contribute to tumor development. A recent study by Vogelstein et al. (Science 1989;244:207-11) demonstrates that allelic deletions are remarkably common in human colorectal carcinomas. In addition, approximately 40% of colorectal cancer also carries K-*ras* mutations at the 12th codon. Heritable human cancer, such as retinoblastoma with well-defined genetic loci, however, is very rare. In order to increase our repertoire of candidate tumor suppressor genes, which may eventually yield to analysis of human cancers, we initiated the present project in an attempt to identify genes which are capable of suppressing the transformed phenotype of NIH 3T3 cells induced by *ras* oncogenes.

Methods Employed:

1. cDNA expression library. Plasmids of three cDNA libraries in mammalian cell-expressing vectors were prepared. A human fibroblast cDNA library was constructed in the pcD_2neo vector; a cDNA library on the π H3M vector was derived from human LAK lymphocytes; and a rat-insulinoma cDNA library was constructed on a retroviral based vector, λ ZD35.

2. Cells. A NIH 3T3 cell transformed by the EJ-*ras* oncogene was twice single-cell cloned to eliminate any flat contaminants. These EJ cells were grown in Dulbecco-modified Eagle's medium supplemented with 10% fetal bovine serum.

3. Transfection. Transfection of a cDNA library to EJ-cells was performed according to the method described by Felgner et al. using a DNA liposome-mediated gene transfer technique. Two days after transfection, cells were trypsinized and re-seeded into 100 mm plates in the growth medium containing G418 (400 μg/ml). The cells were incubated in a CO_2 incubator for 5 days before starting negative selection.

4. Isolation of flat revertants. EJ-cells resistant to G418 (2×10^5) following cDNA transfection were inoculated into a 100 mm plate and incubated for 6 hrs. or overnight in the growth medium. After incubation, the medium was changed to a methionine-deficient medium for negative selection of flat revertants. A selection medium containing fluoro-deoxyuridine ribonucleoside (FUdR) was also used. Cells were incubated for 5 to 6 days (methionine starvation) or for 3 days (FUdR treatment). After each selection, the plates were washed with PBS to eliminate loosely-attached cells, and the remaining cells were trypsinized and replated in 96-well plates in growth medium. Two weeks later, flat clones which appear to be contact-inhibited were isolated for screening.

5. Recovery of cDNA clones from flat revertants. cDNA from flat revertants were recovered as plasmid DNA in E. coli for further analysis.

Major Findings:

The present strategy to clone the suppressor genes is to isolate the morphologically-flat revertants from the EJ-ras transformed NIH 3T3 cells following transfection with a cDNA library containing potential tumor suppressor genes. The functional cDNA clones can then be recovered from the flat revertant cells and further characterized. In a previous study, a flat revertant, R1, has been isolated following mutagenesis of EJ-ras transformed cells. This R1 cell expresses the EJ-ras p21, but is morphologically flat due to presumably cellular mutations. We have used this flat R1 cell in a mixture with known excess of EJ-ras cells to develop a negative selection procedure for isolating flat revertants following transfection with a cDNA library. We found that under methionine starvation most flat R1 cells would survive, while more than 99% of EJ-ras cells died. Therefore, the negative selection procedure using methionine starvation appears to be best in our hands to isolate flat revertants after transfection with a cDNA library. In one transfection experiment with a cDNA library constructed from a human fibroblast, more than 40 morphologically-flat clones have been isolated from 4×10^5 G418-resistant cells which have retained copies of cDNA containing the neo^R genes from the library. Experiments are in progress to characterize these flat revertants and to recover the cDNA clones. Using a similar approach as in this study, Noda et al. have recently succeeded in identifying a candidate suppressor gene, Krev-1, from a flat revertant of K-ras transformed NIH 3T3 cells (Cell 1989;56:77-84).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05595-01 LMO

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

DNA Topoisomerase I Activity in Retroviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	E. Priel	Visiting Scientist	LMO NCI
Others:	D. Blair	Supv. Research Chemist	LMO NCI

COOPERATING UNITS *(if any)*

Ben Gurion University, Beer Sheva, Israel, (S. Segal, M. Abound); Nuc. Acid. & Syn. Lab., PRI, Frederick, MD., (S. Showalter); BRI, Frederick, MD., (M. Roberts, S. Oroszlan)

LAB/BRANCH

Laboratory of Molecular Oncology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.85	0.85	0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In this work, we demonstrate the existence of a novel DNA topoisomerase I (topo I) activity associated with two strains of purified human immunodeficiency virus type-1 (HIV-1), equine infectious anemia virus (EIAV) and Moloney murine leukemia virus (Mo-MuLV). The relaxation activity from these viruses was inhibited by Camptothecin (CPT), a specific topo I inhibitor. The viral-associated topo I activity is Mg++-dependent and possesses other characteristics different from the host-cell enzyme. This topo I activity was removed from the viral lysate by anti-topo I serum. Western blot analysis indicates that an 11.5 Kd protein from both strains of human immunodeficiency virus, type-1 (HIV-1) and an 11 Kd protein from EIAV is recognized by this anti-topo I serum. No similar protein can be detected in the appropriate nuclear or cytoplasmic cell extracts. Moreover, topo I activity was present in isolated and purified EIAV cores. Immunoprecipitation assays using anti-topo I serum and Western blot analysis using anti-p11 EIAV nucleocapsid protein serum indicate that the anti-topo I serum removes the p11 nucleocapsid protein from the EIAV cores, together with topo I activity. These results may suggest that the topo I activity is associated with the p11 nucleocapsid protein found in the EIAV cores.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

E. Priel	Visiting Scientist	LMO NCI
D. Blair	Supv. Research Chemist	LMO NCI

Objectives:

Retroviruses are characterized by their ability to establish persistent infections as a result of integration of their proviral DNA into the host cell genome. The viral replication cycle from intracellular penetration to final integration and expression is undoubtedly dependent on numerous topological changes in the viral and host genomes. Since DNA topoisomerases have been shown to be responsible for the induction of topological changes in DNA and to participate in many vital cellular reactions involving DNA, it was of interest to investigate whether a topoisomerase activity could be demonstrated in retroviral particles.

Methods Employed:

Topoisomerase I was assayed by measuring the relaxation of purified supercoiled plasmids on agarose gels following incubation with viral lysates or viral cores. Standard methods of immunoprecipitation, polyacrylamide gel analysis, and Western blot analysis were used.

Major Findings:

1. We demonstrate the presence of a novel topo I activity in mammalian retroviruses. The topo I activity from HIV-1, EIAV and Mo-MuLV possesses similar characteristics which are different from the enzyme detected in extracts of cells in which the viruses were grown. The relaxation activity of the viral-associated enzymes was Mg^{++}-dependent. This strict Mg^{++} dependence has not been observed for topo I from other eukaryotic sources. The viral-associated topo I was inhibited by 1 mM ATP, but the cellular topo I activity was only weakly inhibited. This inhibitory effect of ATP was not observed with most of the eukaryotic topo I enzymes.

2. Immunoprecipitation assays and Western blot analysis using anti-topo I sera detach an 11 Kd protein from the viral particle. No similar protein can be detected in the appropriate nuclear cell extract. Moreover, the topo I activity is blocked by the anti-topo I serum and not by normal serum.

3. Topo I activity is found in isolated and purified EIAV cores. An 11 Kd protein from the EIAV cores is recognized and could be immunoprecipitated by topo I antibodies. This 11 Kd protein is also recognized by the anti-EIAV p11 (nucleocapsid protein) serum. Immunoprecipitation by topo I antibodies removes the p11 nucleocapsid protein from the supernatant, together with the

topo I activity, and anti-p11 serum recognizes the 100 Kd cellular topo I protein. These results strongly suggest that this 11 Kd protein possesses the topo I activity seen in the retroviral particle, and we are now examining this possibility.

ANNUAL REPORT OF

LABORATORY OF MOLECULAR VIROLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 through September 30, 1989

The Laboratory of Molecular Virology (1) analyzes the mechanism of gene expression in normal and transformed eukaryotic cells; (2) uses viruses as tools for probing cellular regulatory mechanisms; (3) plans and conducts research on transforming proteins to define their properties in normal cells and their potential role in the development of neoplasms; and (4) investigates the mechanisms by which cellular gene expression, viral gene expression, and interactions between viruses may influence the transformation of cells. A summary of some of the major efforts follows:

SV40/Adenovirus

The DNA tumor viruses simian virus 40 (SV40) and adenovirus have provided model systems for the study of eukaryotic gene regulation and oncogenic transformation. The transforming region of the DNA tumor virus SV40 contains coding sequences for three proteins: large T-antigen, small t-antigen and simian virus 40 early leader protein (SELP). SV40 T-antigen acts as a trans-acting transcriptional activator protein to increase expression of viral and cellular genes. Analysis of SV40 T- and t-antigens suggested that both proteins have domains which are similar to regions of the adenovirus E1A protein that are important for transformation, transcriptional activation and transcriptional repression. In view of these homologies, the ability of the SV40 t-antigen to act as a trans-acting regulatory protein was analyzed. Using transfection assays, it was demonstrated that t-antigen acts as a trans-acting transcriptional activator protein, capable of inducing transcription from promoters which are responsive to E1A. Co-transfection of plasmids containing the cDNA sequence for t-antigen with the adenovirus E2A promoter increases expression from the promoter greater than tenfold, similar to the activity observed with E1A. Transcriptional activation by t-antigen is dependent upon the presence of E2A enhancer sequences. Analysis of t-antigen mutants demonstrates that amino acids 112-173, which are unique to t-antigen, are required for activation of the E2A promoter. Using S1 nuclease analysis, it was demonstrated that t-antigen activates transcription from polymerase III promoters. To gain further understanding of the mechanisms by which trans-acting factors regulate gene expression, the regulation of transcription factors which bind to the adenovirus E2A enhancer was examined. Adenovirus E2A upstream sequences which contain the binding sites for ATF and EIIF are sufficient to act as an enhancer in response to both SV40 T/t-antigen and adenovirus E1A. A specific mutation of either the ATF and EIIF binding site demonstrate that both act as positive regulators. Using gel-shift analysis, it was demonstrated that the binding activity of EIIF is increased following adenovirus infection. In contrast, the binding activity of ATF is decreased by adenovirus infection.

HTLV-I

The human T-cell leukemia virus, HTLV-I, has been established as the etiological agent for adult T-cell leukemia. Taxl (40 kD) is a viral coded trans-acting protein which is essential for full transcriptional expression of viral genes and has been shown to be tumorigenic in transgenic mice. Taxl responsive sequences within the HTLV-I LTR have been mapped and cellular transcription factors which interact with these sequences identified. Important findings include: 1) taxl appears to trans-activate responsive LTR elements through the induction of a 180 kD cellular protein; 2) mutational analyses correlate the trans-activation of the HTLV-I LTR by taxl with the presence of a cAMP responsive octonucleotide; 3) two signal transduction agents, cAMP and TPA, are both potent activators of the HTLV-I LTR. Relevant to the last observation, we have defined both the cAMP-responsive and the TPA-responsive sequence elements with the HTLV-I LTR; 4) taxl interacts indirectly, most likely through protein-protein interaction, to taxl responsive sequences in the HTLV-I LTR; and 5) taxl binding to the HTLV-I LTR is mediated by a 36 kD cellular protein.

At this point, little is known about the functional domains of taxl which are important for trans-activation or transformation. The taxl protein binds specifically to a zinc affinity column. The putative metal binding domain of taxl may play an important role in trans-activation. Zinc finger structures have been shown to be important for DNA-protein interactions. In addition, metal binding domains have been shown to be important for protein-protein interactions, such as dimer formation in the human immunodeficiency virus (HIV) tat protein. Since taxl appears not to bind DNA directly, its interaction with cellular transcription factors may be facilitated by coordinating a common zinc ion. Alternatively, the metal binding domain of taxl may allow it to interact with DNA when complexed with a cellular factor. A taxl mutant lacking the first 58 amino acids, thus the metal binding domain, is 85% less effective at trans-activating the HTLV-I LTR than wild-type taxl.

Another important area of the HTLV-I research program involves the development of transgenic mice. Three lines of transgenic mice expressing the HTLV-I taxl gene develop neurofibromas which recruit granulocytes into the mass of transformed cells in vivo. It has been demonstrated that this is the result of tumor secretion of significant quantities of granulocyte-macrophage colony stimulating factor (GM-CSF). This phenomenon is analogous to the recruitment of mast cells into human neurofibromas. In addition to massive infiltration of granulocytes into tumors, systemic effects are also produced including massive splenomegaly, myeloid hyperplasia and peripheral granulocytosis as is seen in patients with adult T-cell leukemia (ATL). Expression of IL-2 receptor is also induced in the neurofibromas which represents the first demonstration that the IL-2 receptor can be produced in vivo in a nonlymphoid cell. This model provides the first in vivo system to study HTLV-I taxl trans-activation.

In addition to ATL, HTLV-I has also been etiologically linked to tropical spastic paraparesis and possibly multiple sclerosis. However, no understanding of the mechanisms leading to this neuropathology has yet emerged. HTLV-I taxl transgenic mice develop neurofibromas of perineural fibroblast origin which

secrete significant quantities of nerve growth factor (NGF). This observation suggests that taxl may stimulate NGF production and may provide important new insights into the mechanism of HTLV-I-induced neurologic disease. Nerve growth factor receptor is also produced in the tumors. Thus, an autocrine mechanism of transformation may result in the formation of neurofibromas. The interaction between taxl and NGF is being studied on a molecular level.

CMV

Human cytomegalovirus (CMV) infection has been associated with several neoplastic diseases, including prostatic carcinoma, adenocarcinoma of the colon, cervical carcinoma, and Kaposi's sarcoma. The morphological transforming region II (mtrII) of CMV Towne has been localized to a 980 base-pair fragment containing three putative open reading frames (ORFs) of 79, 83, and 34 amino acids (aa). Noncoding DNA sequence elements which have the potential to form stem-loop structures were also observed within mtrII. To determine what elements within CMV Towne mtrII are important in transformation, colinear regions in other CMV strains (AD169 and Tanaka) were isolated and a comparison of transforming potential was performed. The results indicated that the 2.2-kilobase colinear region in strain AD169 was transforming, whereas the colinear mtrII region in strain Tanaka showed significantly less transforming potential. Analysis of the nucleotide sequence data of these colinear regions revealed the presence of the 79-aa ORF in strains Towne and AD169 and its absence in strain Tanaka. In addition, Bgl II-digested Towne mtrII, which was cleaved within the 79-aa ORF, was shown to display significantly reduced transforming potential. Since the 83- and 34-aa coding sequences were interrupted in both the transforming AD169 colinear region and the nontransforming Tanaka strains, these ORFs were thought not to be important in transformation. Analysis of the stem-loop structures within each of the mtrII colinear regions did not reveal significant changes among the transforming and nontransforming colinear fragments. Thus, the comparative data indicate an important role for the 79-aa ORF in transformation.

JC Virus

JC virus (JCV) is an oncogenic human papovavirus that has been postulated to be involved in the formation of glial tumors in patients with progressive multifocal leukoencephalopathy (PML). In transgenic mice, the JCV-containing animals developed adrenal neuroblastomas. Virus production in tissue culture is restricted to human fetal glial cells and is regulated at the level of transcription and DNA replication. Oligonucleotides have been synthesized which span the 98 base-pair repeated region of the JCV enhancer. Gel retardation and ultraviolet cross-linking experiments using these oligonucleotides have identified three proteins from human fetal glial cell extracts which associate with this region. Two proteins of 82 kilodaltons (kD) and 80 kD recognize the 5' and 3' regions of the 98 base-pair region, respectively. The molecular weights of these proteins are similar to those of proteins similarly identified from human HeLa cell extracts. One protein specifically binds to the central region of the JCV repeat, but was found to have a molecular weight of 45 kD in human fetal glial cell extracts and 85 kD in human HeLa cell extracts. These proteins, which recognize the essential regions of the enhancer, will be purified and tested for functional activity in the *in vitro* transcription system. In a separate series of experiments,

several cell lines which have been morphologically and biochemically staged to define their development from neural crest cells have been analyzed for their permissiveness to JCV transcription, DNA replication and virus formation. One of these cell lines, designated IN, is permissive for JCV early transcription and viral DNA replication. Of interest, addition of retinoic acid, which stimulates neuronal cell differentiation, elevates the level of both JCV transcription and DNA replication.

Ras

Ras genes are a family of highly conserved genes in evolution and they have been implicated in human cancer. Expression of the ras1 and ras2 genes of Saccharomyces cerevisiae has been examined at the transcriptional and translational levels. We have constructed deletions within the promoter region of the ras2 gene in yeast S. cerevisiae. These deletions map the positive regulatory elements involved in the transcriptional regulation of the ras2 gene. In addition, the promoter deletions result in the synthesis of varying levels of ras2 protein. The accumulation of a minimal amount of ras2 protein is required to initiate the yeast cell cycle START in nonfermentable carbon source. Our previous results had suggested that the ras2 gene product carries out a function in sporulation for which ras1 cannot substitute. The pattern of ras2 protein synthesis in the promoter deletion mutant shows that the hypersporulation phenotype of $Ras1^{+}$ $ras2^{-}$ cells can be overcome by a minimal amount of ras2 protein.

The ras2 protein is phosphorylated in vivo and two different protein kinase activities are involved in the phosphorylation. One of the phosphorylation activities was identified to be the cyclic AMP-dependent protein kinase.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05216-09 LMV

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Ras Oncogene Regulation in Yeast

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ravi Dhar	Visiting Scientist	LMV NCI
Others:	Julianna Lisziewicz	Guest Researcher	LMV NCI
	Nadera Ahmed	Microbiologist	LMV NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
3.0	2.0	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Ras genes are a family of highly conserved genes in evolution and have been implicated in human cancer. Expression of the ras1 and ras2 genes of Saccharomyces cerevisiae has been examined at the transcriptional and translational levels. Deletion mutants within the promoter region of the ras2 gene in yeast S. cerevisiae have been constructed. These deletions map the positive regulatory elements involved in the transcriptional regulation of the ras2 gene. In addition, the promoter deletions result in the synthesis of varying levels of ras2 protein. The accumulation of a minimal amount of ras2 protein is required to initiate the yeast cell cycle START in nonfermentable carbon source. Our previous results had suggested that the ras2 gene product carries out a function in sporulation for which ras1 cannot substitute. The pattern of ras2 protein synthesis in the promoter deletion mutant shows that the hypersporulation phenotype of $Ras1^{+}$ $ras2^{-}$ cells can be overcome by a minimal amount of ras2 protein.

The ras2 protein is phosphorylated in vivo and two different protein kinase activities are involved in the phosphorylation. One of the phosphorylation activities has been identified to be the cyclic AMP-dependent protein kinase.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ravi Dhar	Visiting Scientist	LMV	NCI
Julianna Lisziewicz	Guest Researcher	LMV	NCI
Nadera Ahmed	Microbiologist	LMV	NCI

Objectives:

Our major objectives are:

1. To study the transcriptional and translational regulation of the two *ras* genes in yeast and their extragenic suppressors.

2. To study the phenotype of *ras* mutants and correlate gene regulation with biological function.

Methods Employed:

Recombinant DNA technology, Southern and northern blot analysis, immunoprecipitation, and western blots.

Major Findings:

1. Ras1 and ras2 are differentially regulated.

2. Ras1 is regulated at the transcriptional level.

3. Ras2 is regulated at both transcriptional and translational levels.

4. Ras1 cannot substitute for ras2 protein in sporulation, even when ras1 protein is overproduced.

5. Minimal amounts of ras2 protein are required to overcome the hypersporulation phenotype of ras2$^-$ cells.

5. Cells making a low level of ras2 protein take more time to come out of cell cycle arrest; a minimal amount of ras2 protein needs to accumulate for cells to enter the cell cycle START.

6. Two upstream regulatory elements (UAS) involved in the transcriptional regulation of ras2 genome have been identified.

7. The precursor ras protein is processed through multiple steps before the mature protein is bound to the membranes.

8. Ras2 protein is phosphorylated by at least two different protein kinase activities, one of which is cAMP-dependent protein kinase.

Publications:

Kondaiah P, Van Obberghen-Schilling E, Ludwig LR, Dhar R, Sporn MB, Roberts, AB. cDNA cloning of porcine transforming growth factor-β1 mRNAs: evidence for alternate splicing and tissue specific regulation. J Biol Chem 1988;263:18313-17.

Sreenath TLV, Breviario D, Ahmed N, Dhar R. Two different protein kinase activities phosphorylate ras2 protein in Saccharomyces cerevisiae. Biochem Biophys Res Commun 1988;157:1182-89.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05254-08 LMV

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	John Brady	Acting Chief, VTBS	LMV	NCI
Others:	Susan Marriott	IRTA Fellow	LMV	NCI
	Scott Gitlin	Senior Staff Fellow	LMV	NCI
	Paul Lindholm	Medical Staff Fellow	LMV	NCI

COOPERATING UNITS *(if any)*

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The human T-cell leukemia virus, HTLV-I, has been established as the etiological agent for adult T-cell leukemia. Taxl (40 kD) is a viral coded trans-acting protein which is essential for full transcriptional expression of viral genes and has been shown to be tumorigenic in transgenic mice. At this point, little is known about the functional domains of taxl which are important for trans-activation or transformation. The taxl protein binds specifically to a zinc affinity column. The putative metal binding domain of taxl may play an important role in trans-activation. Zinc finger structures have been shown to be important for DNA-protein interactions. In addition, metal binding domains have been shown to be important for protein-protein interactions, such as dimer formation in the human immunodeficiency virus (HIV) tat protein. Since taxl appears not to bind DNA directly, its interaction with cellular transcription factors may be facilitated by coordinating a common zinc ion. Alternatively, the metal binding domain of taxl may allow it to interact with DNA when complexed with a cellular factor. A taxl mutant lacking the first 58 amino acids, thus the metal binding domain, is 85% less effective at trans-activating the HTLV-I LTR than wild-type taxl.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV	NCI
Susan Marriott	IRTA Fellow	LMV	NCI
Scott Gitlin	Senior Staff Fellow	LMV	NCI
Paul Lindholm	Medical Staff Fellow	LMV	NCI

Objectives:

The goal of this study is to identify functional domains in the transforming protein of HTLV-I, taxl.

Methods Employed:

Transient assay systems, recombinant DNA technology, RNA analysis by hybridization and nuclease protection, purification of the taxl protein, zinc affinity chromotography, western blot.

Major Findings:

1. The HTLV-I taxl protein binds specifically to a zinc affinity column.

2. The HTLV-I taxl mutant lacking the first 58 amino acids, thus the metal binding domain, is 85% less effective in trans-activation of the HTLV-I LTR.

Publications:

Marriott S, Lindholm P, Gitlin SD, Brown KM, Radonovich MF, Duvall JF, Brady JN. Molecular analysis of trans-activation in the HTLV-I LTR. In: Groopman JE, Chen I, Essex M, Weiss R, eds. Early human retroviruses, UCLA Symposia on Molecular and Cellular Biology, Vol. 119. New York: Alan R. Liss (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05391-06 LMV

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transcriptional Analysis of the JC Virus Enhancer

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.:	John Brady	Acting Chief, VTBS	LMV	NCI
Others:	James Remenick	Guest Researcher	LMV	NCI

COOPERATING UNITS *(if any)*

National Institute of Neurological and Communicative Disorders and Stroke, NIH (Eugene Major)

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

JC virus (JCV) is an oncogenic human papovavirus that has been postulated to be involved in the formation of glial tumors in patients with progressive multifocal leukoencephalopathy (PML). In transgenic mice, the JCV-containing animals developed adrenal neuroblastomas. Virus production in tissue culture is restricted to human fetal glial cells and is regulated at the level of transcription and DNA replication. Oligonucleotides have been synthesized which span the 98 base-pair repeated region of the JCV enhancer. Gel retardation and ultraviolet cross-linking experiments using these oligonucleotides have identified three proteins from human fetal glial cell extracts which associate with this region. Two proteins of 82 kilodaltons (kD) and 80 kD recognize the 5′ and 3′ regions of the 98 base-pair region, respectively. The molecular weights of these proteins are similar to those of proteins similarly identified from human HeLa cell extracts. One protein specifically binds to the central region of the JCV repeat, but was found to have a molecular weight of 45 kD in human fetal glial cell extracts and 85 kD in human HeLa cell extracts. These proteins, which recognize the essential regions of the enhancer, will be purified and tested for functional activity in the in vitro transcription system. In a separate series of experiments, several cell lines which have been morphologically and biochemically staged to define their development from neural crest cells have been analyzed for their permissiveness to JCV transcription, DNA replication and virus formation. One of these cell lines, designated IN, is permissive for JCV early transcription and viral DNA replication. Of interest, addition of retinoic acid, which stimulates cell differentiation, elevates the level of both JCV transcription and DNA replication in neuronal cells.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV	NCI
James Remenick	Guest Researcher	LMV	NCI

Objectives:

To identify the transcriptional sequences and the proteins which they bind in the regulation of RNA polymerase II transcription.

Methods:

Recombinant DNA techniques, CAT and Luciferase analyses, in vitro transcription, nucleic acid hybridization, construction of single base-pair and deletion mutations, and DNA transfection.

Major Findings:

1. The 98 base-pair repeat of JCV binds at least three proteins that have been identified in human fetal glial and HeLa cell extracts.

2. The JCV promoter is active in vitro in reactions containing either glial cell or HeLa cell extract; however, only glial cell extracts will produce a properly initiated transcript.

3. Early transcription from the JCV promoter is restricted to certain glial and neuroblastoma type cells that may represent a specific stage of neural development.

4. Retinoic acid, which stimulates cellular differentiation, specifically activates JCV transcription and DNA replication in neuronal cells.

Publications:

Marriott SJ, Brady J. Enhancer function in eukaryotic gene regulation. Biochim Biophys Acta, Cancer Reviews 1989 (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05392-06 LMV

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Regulation of Transcription by Large T-Antigen

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	John Brady	Acting Chief, VTBS	LMV	NCI
Others:	Janet Duvall	BioLab Tech	LMV	NCI

COOPERATING UNITS (if any)

Dana Farber Cancer Institute, Boston, MA (Dr. David Livingston)
Joslin Diabetes Center, Boston, MA (Dr. M. Loeken)

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The transforming region of the DNA tumor virus SV40 contains coding sequences for three proteins: large T-antigen, small t-antigen and simian virus 40 early leader protein (SELP). SV40 T-antigen acts as a trans-acting transcriptional activator protein to increase expression of viral and cellular genes. Analysis of SV40 T- and t-antigens suggested that both proteins have domains which are similar to regions of the adenovirus E1A protein that are important for transformation, transcriptional activation and transcriptional repression. In view of these homologies, the ability of the SV40 t-antigen to act as a trans-acting regulatory protein was analyzed. Using transfection assays, it was demonstrated that t-antigen acts as a trans-acting transcriptional activator protein, capable of inducing transcription from promoters which are responsive to E1A. Co-transfection of plasmids containing the cDNA sequence for t-antigen with the adenovirus virus E2A promoter increases expression from the promoter greater than ten-fold, similar to the activity observed with E1A. Transcriptional activation by t-antigen is dependent upon the presence of E2A enhancer sequences. Analysis of t-antigen mutants demonstrates that amino acids 112-173, which are unique to t-antigen, are required for activation of the E2A promoter. Using S1 nuclease analysis, it was demonstrated that t-antigen activates transcription from polymerase III promoters. To gain further understanding of the mechanisms by which trans-acting factors regulate gene expression, the regulation of transcription factors which bind to the adenovirus E2A enhancer was examined. Adenovirus E2A upstream sequences which contain the binding sites for ATF and EIIF are sufficient to act as an enhancer in response to both SV40 T/t-antigen and adenovirus E1A. A specific mutation of either the ATF and EIIF binding site demonstrate that both act as positive regulators. Using gel-shift analysis, it was demonstrated that the binding activity of EIIF is increased following adenovirus infection. In contrast, the binding activity of ATF is decreased by adenovirus infection.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV	NCI
Janet Duvall	BioLab Tech	LMV	NCI

Objectives:

To identify mechanisms by which eukaryotic genes, transcribed by RNA polymerase II, are regulated at the transcriptional level.

Methods Employed:

Recombinant DNA techniques, in vitro transcription, eukaryotic cell transfection, nucleic acid hybridization, western blot analysis of protein, gel electrophoresis, construction of deletion and point mutants, gel-shift analysis, CAT assays.

Major Findings:

1. A protein that interacts specifically with the SV40 major late upstream control region has been identified. Addition of partially purified protein to in vitro transcription assays decreases the level of SV40 late transcription.

2. SV40 t-antigen acts as a trans-acting regulatory protein to increase expression of both polymerase II and polymerase III genes.

3. Adenovirus E2A regulatory sequences that contain the binding sites for two DNA binding proteins, ATF and EIIF, are sufficient to act as an inducible enhancer in response to SV40 T/t-antigen and E1A. Adenovirus infection results in the modulation of both ATF and EIIF binding activity.

Publications:

Loeken M, Bikel I, Livingston DM, Brady J. Transcriptional trans-activation of RNA polymerase II and III promoters by SV40 small t-antigen. Cell 1988;55:1171-77.

Loeken M, Brady J. The adenovirus EIIA enhancer: analysis of regulatory sequences and changes in binding activity of ATF and EIIF following adenovirus infection. J Biol Chem 1989;264:6572-79.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05394-06 LMV

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Enhancer Elements in B-Lymphocytes and T-Lymphocytes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	John Brady	Acting Chief, VTBS	LMV	NCI
Others:	Michael Radonovich	Biologist	LMV	NCI
	Susan Marriott	IRTA Fellow	LMV	NCI
	Kuan-Teh Jeang	Senior Staff Fellow	LMV	NCI

COOPERATING UNITS (if any)

Institute of Biochemistry, Hungarian Academy of Sciences, Hungary (Dr. I. Boros)
Department of Genetics, George Washington Univ., Washington, D.C. (Dr. K. Brown)

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	3.0	0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The human T-cell leukemia virus, HTLV-I, has been established as the etiological agent for adult T-cell leukemia. The 3' long open reading frame of the human T-cell leukemia virus type-I (HTLV-I) encodes a 40 kD protein (taxI). This protein positively regulates transcription directed by the HTLV-I long terminal repeat (LTR) in a phenomenon known as trans-activation. We have been unable to attribute any sequence-specific DNA binding properties to taxI, suggesting that the protein activates the HTLV-I promoter in an indirect fashion using cellular transcription factors. Our objective is to understand the biochemical mechanism of trans-activation by the taxI protein and the involvement of cellular transcription factors in this process. Important findings include: 1) taxI appears to trans-activate responsive LTR elements through the induction of a 180 kD cellular protein; 2) mutational analyses correlate the trans-activation of the HTLV-I LTR by taxI with the presence of a cAMP responsive octonucleotide; 3) two signal transduction agents, cAMP and TPA, are both potent activators of the HTLV-I LTR. Relevant to the last observation, we have defined both the cAMP-responsive and the TPA-responsive sequence elements with the HTLV-I LTR; 4) taxI interacts indirectly, most likely through protein-protein interaction, to taxI responsive sequences in the HTLV-I LTR; and 5) taxI binding to the HTLV-I LTR is mediated by a 36 kD cellular protein.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV NCI
Michael Radonovich	Biologist	LMV NCI
Susan Marriott	IRTA Fellow	LMV NCI
Kuan-Teh Jeang	Senior Staff Fellow	LMV NCI

Objectives:

This project is focused on understanding the mechanism of action of the HTLV-I taxl protein.

1. Purification of cellular proteins that interact with the HTLV-I LTR.

2. Definition of cellular pathways for the activation of the HTLV-I LTR.

3. Mutagenesis of the HTLV-I taxl-responsive elements.

Methods Employed:

Recombinant DNA techniques, bacterial expression vectors, somatic cell fusion techniques, protein purification, gene expression using transient and permanent assays, and RNA and protein analysis.

Major Findings:

1. Two signal transduction pathways, cAMP and TPA, activate the expression of the HTLV-I LTR. The cAMP-responsive element is contained within three repeated octomeric sequences found in the HTLV-I LTR. The TPA-responsive sequence is contained within two 51 bp elements.

2. The binding activity of a cellular 180 kD protein correlates with the biological trans-activation properties of taxl.

3. The HTLV-I 21 bp taxl-responsive element has been extensively mutagenized to define the minimally essential core nucleotides necessary for biological response.

4. HTLV-I taxl binds indirectly, most likely through protein-protein interaction, to taxl responsive sequences in the viral LTR.

Publications:

Jeang K-T, Boros I, Radonovich M, Duvall J, Khoury G, Brady J. Cellular proteins and DNA sequences involved in trans-activation of the HTLV-I LTR by taxl. In: Cullen B, Franza R, Wong-Staal F, eds. HIV and HTLV-I gene expression, Banbury Conference. New York: Cold Spring Harbor Laboratory, 1988;265-80.

Jeang K-T, Boros I, Brady J, Radonovich M, Khoury G. Identification of cellular factors that bind to the 21 bp $p40^{x}$-responsive region in the HTLV-I LTR. J Virol 1988;62:4499-509.

Jeang K-T, Shank PR, Kumar A. Transcriptional activation of homologous viral LTR by the HIV-I or the HTLV-I tat proteins occurs in the absence of de novo protein synthesis. Proc Natl Acad Sci USA 1988;85:8291-5.

Marriott SJ, Boros I, Duvall JF, Brady JN. Indirect binding of HTLV-I taxl to a responsive element of the viral LTR. Mol Cell Biol (In Press).

Marriott SJ, Lindholm PF, Gitlin SD, Brown KM, Radonovich MF, Duvall JF, Brady JN. Molecular analysis of trans-activation in the HTLV-I LTR. In: Groopman JE, Chen I, Essex M, Weiss R, eds. Early human retroviruses, UCLA Symposia on Molecular and Cellular Biology, Vol. 119. New York: Alan R. Liss (In Press).

Radonovich M, Jeang K-T. Activation of the HTLV-I LTR by TPA (12-O-tetradecanoyl-phorbol-13-acetate) and by tax (p40x) occurs through similar but functionally distinct target sequences. J Virol 1989;63:2987-2994.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05605-01 LMV

PERIOD COVERED
October 1, 1988 through September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Transformation by Human CMV

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.: John Brady Acting Chief, VTBS LMV NCI

COOPERATING UNITS *(if any)*

Georgetown Medical Center, Washington, D.C. (Dr. Leonard Rosenthal)

LAB/BRANCH
Laboratory of Molecular Virology

SECTION
Virus Tumor Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.25	0.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human cytomegalovirus (CMV) infection has been associated with several neoplastic diseases, including prostatic carcinoma, adenocarcinoma of the colon, cervical carcinoma, and Kaposi's sarcoma. The morphological transforming region II (mtrII) of CMV Towne has been localized to a 980 base-pair fragment containing three putative open reading frames (ORFs) of 79, 83, and 34 amino acids (aa). Noncoding DNA sequence elements which have the potential to form stem-loop structures were also observed within mtrII. To determine what elements within CMV Towne mtrII are important in transformation, colinear regions in other CMV strains (AD169 and Tanaka) were isolated and a comparison of transforming potential was performed. The results indicated that the 2.2-kilobase colinear region in strain AD169 was transforming, whereas the colinear mtrII region in strain Tanaka showed significantly less transforming potential. Analysis of the nucleotide sequence data of these colinear regions revealed the presence of the 79-aa ORF in strains Towne and AD169 and its absence in strain Tanaka. In addition, Bgl II-digested Towne mtrII, which was cleaved within the 79-aa ORF, was shown to display significantly reduced transforming potential. Since the 83- and 34-aa coding sequences were interrupted in both the transforming AD169 colinear region and the nontransforming Tanaka strains, these ORFs were thought not to be important in transformation. Analysis of the stem-loop structures within each of the mtrII colinear regions did not reveal significant changes among the transforming and nontransforming colinear fragments. Thus, the comparative data indicate an important role for the 79-aa ORF in transformation.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady Acting Chief, VTBS LMV NCI

Objectives:

To genetically map transforming regions of CMV.

Methods Employed:

Tissue culture; DNA transfection; radiolabeling of DNA, RNA and protein; DNA cloning; polyacrylamide gel electrophoresis; Southern blotting; northern blotting and hybridization; S1 nuclease; transformation assays; and DNA sequencing.

Major Findings:

1. CMV morphological transforming region II (mtrII) has been localized to a 980 bp fragment containing three putative open reading frames of 79, 83 and 34 amino acids.

2. DNA sequence analysis of wild-type CMV Towne and transformation-detective CMV Tanaka suggests an important role for the 79-amino acid protein in transformation.

Publications:

Jahan N, Razzaque A, Brady JN, Rosenthal LJ. Human cytomegalovirus mtrII colinear region in strain Tanaka is transformation defective. J Virol 1989;63:2866-9.

Razzaque A, Jahan N, Jariwalla R, Jones C, Brady J, Rosenthal LJ. Localization and sequence analysis of the transforming domain mtrII of human cytomegalovirus. Proc Natl Acad Sci USA 1988;85:5709-13.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05606-01 LMV

PERIOD COVERED: October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.): Sjögren's Syndrome in HTLV-I Transgenic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	John Brady	Acting Chief, VTBS	LMV	NCI
Other:	Jeffrey Green	Biotechnology Fellow	LMV	NCI

COOPERATING UNITS (if any)

American Red Cross, Rockville, MD (Gilbert Jay)
University of California at Davis (Steve Hinrichs)

LAB/BRANCH: Laboratory of Molecular Virology

SECTION: Virus Tumor Biology Section

INSTITUTE AND LOCATION: NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

HTLV-I is a retrovirus etiologically associated with adult T-cell leukemia, tropical spastic paraparesis and possibly multiple sclerosis in humans. It has been documented that certain HTLV-I-infected patients develop Sjögren's syndrome, a disease of presumed autoimmune etiology resulting in severe dysfunction of the lacrimal and salivary glands. Three founder lines of transgenic mice carrying the HTLV-I tax1 gene have been extensively studied and have been shown to develop neurofibromas as well as pathology very similar to that seen in patients with Sjögren's syndrome. Further characterization of these transgenic mice offers the opportunity to study the mechanisms resulting in this disease process and may provide new insights into understanding autoimmunity.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV	NCI
Jeffrey Green	Biotechnology Fellow	LMV	NCI

Objectives:

To study the association between HTLV-I and Sjögren's syndrome.

Methods Employed:

Mouse embryo injection, embryo transfer to pseudopregnant female mice, Southern and northern hybridization, immunofluorescence, immunoprecipitation, in situ hybridization, and histopathology.

Major Findings:

1. Tax is expressed in the ductal epithelial cells of the salivary and lacrimal glands of transgenic mice carrying the HTLV-I taxl gene.

2. Proliferation of ductal cells occurs in the submandibular, sublingual, parotid and lacrimal glands and proliferation is related to the level of tax production.

3. Lymphocytic infiltration of the salivary glands is a late occurrence. Ultimately the acini are destroyed and replaced by lymphocytes and hyalin. This lesion closely resembles that seen in human Sjögren's syndrome. This study provides the first direct evidence that HTLV-I can produce a lesion resembling Sjögren's syndrome.

Publications:

Green JE, Vogel J, Jay G. HTLV-1 transgenic mice: a model for studying viral pathogenesis. In: Roman GC, Vernant JC, eds. HTLV-I and the nervous system. New York: Alan R. Liss 1989;589-93.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05607-01 LMV

PERIOD COVERED

October 1, 1988 through September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Activation of Cellular Genes in HTLV-I Transgenic Mice

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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LAB/BRANCH

Laboratory of Molecular Virology

SECTION

Virus Tumor Biology Section

INSTITUTE AND LOCATION

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.5	1.5	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In vitro studies have demonstrated that the tax1 gene of human T-cell leukemia virus, type 1 (HTLV-I) is capable of trans-activating several cellular genes which may be important for HTLV-I-induced transformation leading to adult T-cell leukemia (ATL). Three lines of transgenic mice expressing the HTLV-I tax1 gene develop neurofibromas which recruit granulocytes into the mass of transformed cells in vivo. It has been demonstrated that this is the result of tumor secretion of significant quantities of granulocyte-macrophage colony stimulating factor (GM-CSF). This phenomenon is analagous to the recruitment of mast cells into human neurofibromas. In addition to massive infiltration of granulocytes into tumors, systemic effects are also produced including massive splenomegaly, myeloid hyperplasia and peripheral granulocytosis as is seen in patients with ATL. Expression of the IL-2 receptor is also induced in the neurofibromas which represents the first demonstration that the IL-2 receptor can be produced in vivo in a nonlymphoid cell. This model provides the first in vivo system to study HTLV-I tax1 trans-activation.

HTLV-I has been etiologically linked to tropical spastic paraparesis and possibly multiple sclerosis. However, no understanding of the mechanisms leading to this neuropathology has yet emerged. HTLV-I tax1 transgenic mice develop neurofibromas of perineural fibroblast origin which secrete significant quantities of nerve growth factor (NGF). This observation suggests that tax1 may stimulate NGF production and may provide important new insights into the mechanism of HTLV-I-induced neurologic disease. This result suggests that neurologic disease may result from HTLV-I infection of perineural cells which cause perturbated secretion of factors which may lead to neurologic dysfunction. Nerve growth factor receptor is also produced in the tumors. Thus, an autocrine mechanism of transformation may result in the formation of neurofibromas. The interaction between tax1 and NGF is being studied on a molecular level.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

John Brady	Acting Chief, VTBS	LMV	NCI
Jeffrey Green	Biotechnology Fellow	LMV	NCI
G. Glen Begeley	Medical Staff Fellow	MB	NCI
D. Wagner	Medical Staff Fellow	MB	NCI
T. A. Waldmann	Chief	MB	NCI

Objectives:

To study hematologic growth factors and receptors induced in HTLV-I transgenic mice. To determine whether cells expressing HTLV-I taxl secrete factors which affect nerve function.

Methods Employed:

Mouse embryo injection, embryo transfer to pseudopregnant female mice, Southern and northern hybridization, *in vitro* colony stimulating assays, immunoprecipitation, histopathology, immunocytochemistry, ELISA, functional assays for nerve growth factor and nerve growth factor receptor.

Major Findings:

1. HTLV-I taxl transgenic mice develop neurofibromas of perineural cell origin which express taxl.

2. Neurofibromas in the transgenic mice recruit large quantities of granulocytes unrelated to tumor necrosis. In addition, significant granulocytosis and splenomegaly with myeloid hyperplasia occurs in the mice. These findings correlate with taxl-induced secretion of granulocyte-macrophage colony stimulating factor (GM-CSF) by the tumors.

3. IL-2 receptor is produced by neurofibromas occuring in HTLV-I taxl transgenic mice. This is the first evidence that IL-2 receptor can be induced *in vivo* in a nonlymphoid cell.

4. HTLV-I taxl is expressed and, therefore, neurotropic for perineural fibroblasts in transgenic mice.

5. Taxl expressing neurofibromas produce nerve growth factor receptor and nerve growth factor. This suggests that an autocrine mechanism may be involved in the transformation process.

6. Chronic overproduction of nerve growth factor (NGF) by perineural fibroblasts may lead to neurologic dysfunction. This observation could suggest a new mechanism for HTLV-I-induced neurologic disease.

Publications:

Green JE, Vogel J, Jay G. HTLV-I transgenic mice: a model for studying viral pathogenesis. In: Roman GC, Vernant JC, eds. HTLV-I and the nervous system. New York: Alan R. Liss 1989;589-93.

ANNUAL REPORT OF

THE LABORATORY OF TUMOR CELL BIOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 to September 30, 1989

The objectives of the Laboratory of Tumor Cell Biology (LTCB) are to develop, implement, and analyze data obtained from studies of cellular proliferation, cell differentiation, and biochemical growth characteristics of normal and malignant mammalian cells both in vivo and in vitro. Particular attention is given to leukemogenesis and immune deficiency. Because of unusual access to human blood cells and because of the interest of this group in retroviruses and human herpesvirus (HHV-6), there is special focus on human leukemias and lymphomas, and acquired immunodeficiency syndrome (AIDS). It is anticipated that an enhanced understanding of cell regulatory mechanisms will permit the optimal use of antitumor agents in the therapy of cancer and AIDS.

The Laboratory of Tumor Cell Biology is concerned with several biological and biochemical problems: 1) studies on the cellular and molecular origin and pathogenesis of human leukemia. Biochemical control mechanisms involved in cell differentiation and neoplastic transformation are examined. Tumor viruses of animals are used both as tools as well as for help in understanding mechanisms of naturally occurring animal leukemias and AIDS. Also, studies designed to determine the distribution of human T lymphotropic retroviruses in T-cell leukemia patients, patients with AIDS, and normals in different parts of the world are being carried out; 2) studies on the biochemical events preceding mitosis. An understanding of these events appears essential to the control of proliferation, and information derived from such studies may lead to more effective inhibitors of neoplastic cell growth. Events leading to mitosis are also of interest since many of the effective antitumor agents are useful only when cells are in DNA replication or in mitosis; 3) attempts to develop new approaches to cancer chemotherapy and antiviral agents for treatment of AIDS using information gained from basic cellular studies. In addition, several approaches are being explored to obtain a vaccine against the AIDS virus; 4) studies on the development of biochemical and immunological markers for malignant cells are carried out. Biochemical and immunological studies are also conducted in individuals with disorders associated with an increased incidence of neoplasia; 5) controls regulating cellular growth and differentiation, and the process of malignant transformation in hematopoietic cells; and 6) growth factors (and their receptors) that control the growth and differentiation of blood cells have been isolated and are under intensive study.

Progress made in the past year in various phases of this research effort is summarized below:

Kaposi's Sarcoma (KS)

Spindle-like cells were successfully cultured for long periods of time from the involved tissue specimen taken from the lung, oral cavity, skin, orbit of the eye, tonsil, and pleural effusion obtained from AIDS patients with disseminated

KS. The primary characteristics of these cells have been reported. Further characterization is in progress. Functional studies of these cells with respect to their role in matrix formation or destruction with the help of collagenases, chemotactic and chemoinvasion capabilities have just been completed. The apparent conclusion of this study seems to be that these cultured cells are abnormal smooth muscle cells of vascular origin. These cells have been cultured with the help of a novel growth factor obtained from a T4+ transformed cell line developed by the infection of human umbilical cord blood leukocytes by human T-lymphotropic virus (HTLV-II). Purification of this factor is in progress. The effort to develop reagents is also in progress. These efforts, at present, are limited to the development of monoclonal and monospecific polyclonal antibodies to the long-term cultured AIDS-KS cells. Several monoclonal antibodies have been developed that react only with the AIDS-KS cells and not with the normal human umbilical vein endothelial cells or several other cell types.

The AIDS-KS cells are being further examined for the extent and type(s) of soluble mediators being expressed by them. As was expected and published in the previously mentioned studies, several soluble biologically active mediators have been identified, viz., IL-1B, TNF, TGFB, aFGF and bFGF. In addition, a new factor has also been identified through a collaborative study. This factor is in the process of purification.

These long-term cultured AIDS-KS cells have also been the subject of molecular analyses. These studies were basically directed at the identification of messages for the well-defined factors and, initially, to determine the extent of expression of proteins. In our continuing effort to biologically evaluate the AIDS-KS cells and their in vitro and in vivo functions, we discovered that these cells not only respond preferentially to the T4+ cell-derived factor supplement, but this effect is significantly enhanced by corticosteroids. This is universal for all the AIDS-KS cells. The study for the in vitro part has recently been completed. Since the initial success with a few specimens of lung biopsies and pleural fluids, we have expanded this to include KS specimens from several other sites, such as orbit, buccal cavity, tonsils and skin.

The in vivo systems that were used in previous studies have also been further improved. The use of whole egg for chicken chorioallantoic membrane assay has been discontinued. This was cumbersome, needed special facilities, and was difficult to document. This has been replaced by the "cracked egg" technique which gives us the optical clarity of the petri dish and a flat chicken. This procedure is one of the state-of-the-art techniques used to assess angiogenesis induced by the effector molecule. The other in vivo assay which we developed previously and continue to use is the NCr nude mouse assay. We are now developing a "hairless" guinea pig model for the same purpose. This is much less expensive and gives us the advantage of testing several points (such as titration) on one animal. This effort is still in the preliminary stages but appears promising. We are standardizing the system for the use of special sponges for sustained signal release. These signals, in turn, will induce angiogenesis and other effects, resulting in a developing KS-like lesion.

In addition to the above mentioned studies, a number of promising compounds (with proven anti-angiogenesis capabilities) are being tested. These include both seemingly toxic compounds, such as tetrahydrocortisone as well as nontoxic compounds such as SPPG. This effort is aimed at possible clinical application of

a concept put forward in our previously published work. The hairless guinea pig will be very helpful in this phase of our work.

Human Herpesvirus-6:

The isolation of the novel human herpesvirus (human B lymphotropic virus [HBLV] or HHV-6) from patients with a variety of hematological disorders was first reported by us in 1986. While the concomitant infection by human immunodeficiency virus type-1 (HIV-1) and HHV-6 clearly could be coincidental, other observations suggest a possible synergistic role for HHV-6 in AIDS. In vitro studies have shown that HHV-6 can infect a number of other cell types. For example, we reported the infection of fresh peripheral blood lymphocytes with surface antigen. The infection results in a dramatic cytopathic effect on these cells. Furthermore, a number of cell lines consisting of T- and B-lymphocytes, as well as cells of other origins, e.g., megakaryocytes, could also be infected by HHV-6. Many of these same cell lines are also susceptible to infection by HIV-1. The dual infection of a susceptible cell line by HIV-1 and HHV-6 results in an accelerated loss of viable cells. While these observations are consistent with a possible synergistic activity, the actual mechanisms involved are unknown. HHV-6 could contribute directly by stimulating HIV-1 expression or by interacting with HIV-1 long terminal repeat affecting virus expression.

We have continued to study HHV-6. In addition to the development of information in the areas of biology and molecular biology, which have been reported, the following significant developments have taken place: 1) determination of the role of HHV-6 in lymphoproliferative diseases and chronic fatigue syndrome; 2) study of the interaction of HHV-6 and HIV-1 and HIV-2 in lymphoid cells (T4+) to understand the role of this virus, if any, in AIDS; and 3) a collaborative study was initiated for developing a series of monoclonals to HHV-6 proteins.

The role of HHV-6 as a primary etiological agent or as a cofactor in human disease was investigated. Recent findings show that approximately 14% of the heterophile-negative infectious mononucleosis (IM) cases were positive for IgM antibody for HHV-6 virus capsid antigen (VCA) only. The particular role of HHV-6 in chronic fatigue syndrome (CFS) patients is still under investigation. However, 51% of the patients had elevated antibody to HHV-6 VCA as compared to age- and sex-matched healthy donors. Active HHV-6 infection was detected in 9/12 CFS patients' peripheral lymphocytes, as directed by HHV-6 monoclonal antibody. Normal donor lymphocytes (1/11) contained HHV-6 VCA-positive cells. These data are supported by in situ hybridization using HHV-6 DNA as a probe. Dual infection with HHV-6 and HIV-1 or HIV-2 of CD4+ cells (fresh cells or cell lines) showed enhanced killing, a significant increase in HIV-1 reverse transcriptase (RT) activity, and transactivation of HIV long terminal repeats (LTRs), suggesting that HHV-6 may contribute directly or indirectly to the depletion of CD4+ T-cells in AIDS. An antigen capture assay has recently been developed as a consequence of the purification of several viral proteins and monoclonal antibodies. We need to test a reasonable panel of sera/body fluids to determine the levels of viral antigen in specific disease groups and normal people.

Persistence and Modulation of HIV-1 Expression in Cells of Mononuclear Phagocyte Lineage

In our previous studies we have shown that the monocyte/macrophages (M/M) are highly susceptible and permissive host cells for HIV-1. This cell system can be

successfully applied for virus isolation in situations when the T-cell system fails. Using peripheral blood (PB)-derived M/M as targets for HIV-1, the virus was recovered from brain, peripheral blood, bone marrow, lung and skin specimens taken from HIV-1-infected individuals at various stages of disease development. Over 30 HIV-1 isolates have been obtained and most of them characterized by nucleic acid hybridization analysis. It has been successfully demonstrated that in some cases of early infection with the virus (antibody-negative, acute infection) or in clinically asymptomatic antibody-positive individuals, HIV-1 could be recovered only from M/M (e.g., HTLV-III_B infection of a laboratory worker). In addition, the M/M system enabled us to isolate and study the so called "low" replicative types of HIV-1. Three such isolates have been obtained: one from esophageal tissue of a patient with acute HIV-1 infection ($HTLV\text{-}III_{RM\text{-}eu}$), one from brain ($HTLV\text{-}III_{CG\text{-}br}$) and one from thymic tissue ($HTLV\text{-}III_{CG\text{-}thy}$) of a pediatric case with full-blown AIDS.

In another study virus has been isolated from PB M/M from seronegative contacts of HIV-1-infected individuals (e.g., spouses, cohorts) but not T-cells. Nucleic acid analysis of these new isolates has shown them to have unique banding patterns when hybridized to molecular probes of HIV-1.

Studies are continuing to define the level of HIV-1 expression in M/M infected with these "low" replicative isolates as compared to "high" replicative isolates. HIV-1 expression was followed by in situ hybridization and by RT assay. Using the same multiplicity of HIV-1 infection, it was shown that there was at least a tenfold difference in virus production between "low" and "high" replicative isolates. To delineate which factors can modulate HIV-1 expression, PB-derived M/M infected with low replicative isolates were exposed to several cytokines. Unlike the case of the model system of HIV-1-infected U-937 neoplastic cells, culture fluids harvested from phytohemagglutinin (PHA)-stimulated T-cells and mixed lymphocyte reaction (MLR) had no effect on HIV-1 expression in M/M infected with "low" replicative HIV-1 isolates. These results suggest that observations obtained from the model system of HIV-1 neoplastic T- or monocytic cell lines may have limited application for in vivo situations of HIV-1-natural host cell interaction. In contrast, using colony stimulating factors (CSF), both the purified CSF as well as the recombinant CSF, accelerated the virus expression in M/M which were infected with "low" replicative HIV-1 isolates and cultured in the presence of these lymphokines. In addition, CSF produced by giant cell tumor (GCT) not only accelerated, but actually increased, HIV-1 production in M/M infected with these "low" replicative isolates. However, a single and most effective factor for significant increase of HIV-1 replication in a given M/M infected with "low" replicative HIV-1 isolates was the number of transmissions of a given viral progeny into recipient cells. Conversion of the "low" replicative $HTLV\text{-}III_{CG\text{-}br}$ isolate into a "high" replicative one occurred within four in vitro passages of the propagated viral progeny in M/M. This increased production of the $HTLV\text{-}III_{CG\text{-}br}$ isolate was accompanied by the appearance of multinucleated giant cells. Thus, this system of "low" replicative HIV-1 isolates propagated in PB-derived M/M enables us to study important parameters of virus-host cell interactions essential for our understanding of HIV-1 pathogenesis, namely, heterogeneity of HIV-1 isolates and the dynamics (conversion from "low" to "high" production) with respect to cytopathic effect(s) exhibited on host cells.

Identification of the M/M Receptor for HIV-1

The CD4 receptor is the binding site of HIV-1 on the T-cell. Preliminary binding studies of the virus to U-937, a neoplastic promonocytic cell line, have implicated CD4 as the receptor binding site. We are currently studying the receptor binding of HIV-1 in normal PB M/M using monoclonal antibodies against different epitopes of the CD4 receptor to block infection.

Identification of DNA Sequences Within the HIV-1 Genome Responsible for Efficient Infection and Replication of HIV-1 in PB-Derived M/M

In our previous studies we have quantitatively characterized several HIV-1 isolates for their capacity to infect T-cells and M/M. There were significant differences in ED_{50} between different HIV-1 isolates. For instance, $HTLV\text{-}III_{BA\text{-}L}$ recovered from and propagated only in M/M productively infected these cells at multiplicities of infection (MOI) of 0.5 to 1 X 10^2 cpm/ml of RT activity. In contrast, the prototype $HTLV\text{-}III_B$, which readily infects T-cells at these low MOIs, requires an MOI of 5 X 10^5 cpm RT activity/ml to PB-derived M/M. These 1000 to 10000-fold differences between $HTLV\text{-}III_{BA\text{-}L}$ and $HTLV\text{-}III_B$ in capacity to infect M/M are sufficient to permit definitive testing of recombinants generated from these two isolates. In collaboration with Dr. M. Reitz, Jr. (Z01CP05538-03 LTCB), a 4.2 Kb Hind-III fragment of $HTLV\text{-}III_{BA\text{-}L}$ containing tat, trs, env and a portion of 3' orf has been cloned and sequenced. Nucleic acid sequence analyses have suggested that the 4.2 Kb fragment of the $HTLV\text{-}III_{BA\text{-}L}$ (M/M "tropic") isolate does not contain a characteristic portion within the viral genome which would be specifically responsible for efficient infection and replication of the $HTLV\text{-}III_{BA\text{-}L}$ isolate in M/M. Data from infection of T-cells and M/M with hybrid viruses generated from the $HTLV\text{-}III^B$ and $HTLV\text{-}III_{BA\text{-}L}$ isolates, along with data from sequencing of the vDNA (Hind-III fragment), suggest that minor nucleic acid sequences distributed throughout the whole HIV-1 genome most likely are involved in allowing the efficient replication of an HIV-1 isolate in M/M. Recently, a 6.4 kb EcoRI fragment containing a majority of the envelope region of $HTLV\text{-}III_{BA\text{-}L}$ has been cloned and sequenced. Hybrids of this portion of the genome and HTLV-III are being constructed to infect normal T-cells and M/M. Additional information about the genome of M/M tropic isolates is being generated by polymerase chain reaction (PCR). The LTR and a small portion of the GAG region have been sequenced and in the near future constructs will be tested as described previously. Hopefully these experiments will reveal control regions in the HIV-1 genome that will show why certain HIV-1 isolates are T-cell tropic while others are M/M tropic.

Inhibitors of HIV-1 Replication

Several drugs have been examined either alone or in combination for inhibition of HIV-1 replication in cell cultures. Drugs that inhibit virus replication by mechanisms other than reverse transcriptase inhibition and DNA synthesis chain termination may be useful in the treatment of patients with AIDS and AIDS-related complex (ARC).

D-penicillamine (DPA) which presumably interacts with sulfhydryl group-containing proteins, blocks HIV-1 replication in cell cultures. DPA has been used in the past for the treatment of Wilson's disease, chronic hepatitis and rheumatoid arthritis. This compound acts by inhibiting HIV-1 tat protein expression which

is a zinc-containing sulfhydryl protein. A limited clinical trial in AIDS patients is currently in progress.

Amphotericin B methyl ester (AME), a water-soluble derivative of amphotericin B, is a member of the polyene macrolide group of antifungal antibiotics, which interacts with sterols and irreversibly binds to them. It is known to be active against a variety of lipid-enveloped RNA and DNA viruses, several oncogenic retroviruses and different strains of herpesviruses. This antiviral property of AME prompted us to examine its activity against HIV-1, which is also a lipid-enveloped retrovirus. The binding of AME to cholesterol in the membrane of cells causes changes in cell permeability and function, and its binding to sterols of lipid-enveloped viruses causes loss of their infectivity. Both amphotericin B and AME have been found to be potent inhibitors of HIV-1 replication in cell culture (freshly infected and chronically infected) without any toxic side effects. A combination of AME with several other drugs including foscarnet, 3'-azido-3'-deoxythymidine (AZT), 1'-3'-dideoxycytidine (DDC), and dextran sulfate showed both additive and synergistic effects; and it appears that combination therapy may be more effective in the treatment of AIDS patients. Both amphotericin B and nystatin as well as their liposomal encapsulated preparations have been found to be effective in inhibiting HIV-1 replication in freshly infected and chronically infected cells.

Avarol and AZT which inhibit viral replication by different mechanisms, were used to study the role of cytoplasmic factors in independent regulation of host cell and viral gene expression. Both drugs were found to inhibit viral replication and synthesis of virus-encoded protein in a synergistic manner, while at cytostatic drug concentrations, both compounds act antagonistically. Adenosine triphosphate (ATP)-induced transport of viral messengers from isolated nuclei is enhanced by total cytosolic protein from HIV-1-infected cells.

The inhibition of HIV-1 replication by the use of antisense oligonucleotides (synthetic oligonucleotides), which inhibit virus replication by competition hybridization, has been utilized. Since the complete nucleotide sequence of the HIV-1 genome is known, 20 different target sites were selected for the preparation of antisense oligomers. Target sites were selected based on their potential capacity to block recognition functions during viral replication. Antisense oligonucleotides of chain length 20, complementary to sites within or near the sequence repeated at the ends of retrovirus RNA (R region) and to certain splice sites, were most effective. Oligomer derivatives such as thiophosphates, morpholidates, methylphosphonates and oligomers containing cholesterol residues were also found to be very effective inhibitors of HIV-1 replication in both freshly infected and chronically infected cells. In addition, a combination of several of these oligomers showed both an additive as well as synergistic effect. Safety and toxicity studies in mice show that these compounds are completely safe and nontoxic. The inhibitory activity of antisense oligomers containing methylphosphonate residues was found to be dependent on both chain length and the number of phosphonate residues. Introduction of 18 phosphonate groups in an oligomer of chain length 20 significantly increased HIV inhibitory activity relative to the parent oligonucleotide, whereas incorporation of five methylphosphonate residues showed little or no increase in HIV inhibition capacity.

Use of Synthetic Peptides as Vaccines for AIDS

Several approaches to the development of an AIDS vaccine are being pursued in different laboratories. They include the envelope glycoprotein, vaccinia vector, anti-idiotypes, and synthetic peptides as sources for an AIDS vaccine. We have explored the potential usefulness of synthetic peptides as candidate AIDS vaccines, especially a peptide generated from the p17 sequence of HIV-1. The p17 protein was recently found, by electron microscopy using immunogold labeling techniques, by Gelderblom et al. (Max Planck Institute, Berlin, FRG; unpublished results) to be associated with the envelope glycoproteins of HIV-1. Similar conclusions have been drawn by computer modeling techniques. This observation suggests that the HIV-1 p17 epitope may be exposed on the surface in the virions. Hence, gag gene products are important in any consideration of a potential vaccine for AIDS. In addition, because of the genetic variability in the gp120 region of different HIV-1 isolates, which show as much as a 20% divergence in the amino acid sequence of gp120, it is important to look for other approaches to the development of an AIDS vaccine.

A 30 amino acid peptide analog of HIV-1 p17 (termed HGP30) and other analogs spanning the HIV-1 p17 amino acid sequence have been synthesized that cross-react in the HIV-1 p17 radioimmunoassays (RIA) and can be identified by HIV-1 p17 monoclonal antibodies by enzyme-linked immunosorbent assays (ELISA) and Western blotting. Antibodies prepared against HGP30 inhibit HIV-1 replication in cell culture. These studies indicate that HIV-1 p17 may be important in the cell-mediated immunity and that these antibodies may be protective against HIV-1 infection. Pepscan analysis with overlapping non-peptides derived from the sequence of HIV-1 p17 HGP30 identified the sequence (KE) ALDKIEE (EQ) as the major antibody binding site. Sera of 9% of AIDS patients (7/76) and 18% of HIV-1 seropositive healthy homosexuals (40/223) were positive for HGP30 antibodies. Decline in HIV-1 p17 antibodies has been shown to be related to disease progression in both children and adults, suggesting that HIV-1 p17 antibodies may be protective. A limited clinical trial with HGP30 is currently in progress in England.

Tropical Spastic Paraparesis (TSP) and Neurological Dysfunction

Adult T-cell leukemia (ATL) and TSP are diseases that are prevalent in Jamaica and the Caribbean, areas which are endemic for HTLV-I. HTLV-I antibodies have been identified in a large number of patients with TSP. TSP is a neurological syndrome in which patients progressively develop difficulty in walking, stiffness of legs and back pain. A similar disease called HTLV-I-associated myelopathy (HAM) has recently been described in Japan. We have isolated HTLV-I strains from the CSF and PB lymphocytes of TSP patients from Jamaica and Colombia. The HTLV-I strain from CSF of a TSP patient shows differences in the prototype HTLV-I isolate of ATL patients, by restriction enzyme mapping, suggesting that the TSP HTLV-I strain is similar to but not identical to the prototype HTLV-I isolate. A genomic library of this HTLV-I strain has been obtained and is being subcloned for further characterization. Several cell lines developed from TSP patients have been characterized phenotypically by using a panel of monoclonal antibodies to lymphoid cell surface markers, such as CD3, CD4, CD8, CD25, and HLA-DR.

Three of the TSP cell lines and two ATL cell lines have been adapted to total serum-free medium and the supernatants are being used to determine the secretion of some major interleukins and soluble interleukin-2 (IL-2) receptor. The

messenger ribonucleic acid (mRNA) from these cell lines will also be examined for the presence of mRNA for various interleukins and the IL-2 receptor.

Analysis of HIV-1 Reverse Transcriptase Maturation Process

Identification and characterization of HIV-1, HIV-2, and SIV gag-pol precursors: HIV synthesizes a gag-pol fusion protein which is later cleaved to give mature gag and pol proteins. The identification and analysis of such a precursor has been limited until now by the low amount of protein that can be recovered from infected cells or mature virions. With the activity gel analysis we have shown the presence of 165 KDa (HIV-1) and 170 KDa (HIV-2, SIV) catalytically active bands that may represent the entire product of the gag-pol gene. Studies are in progress to obtain large quantities of the precursor for further characterization by (a) construction of an HIV-1 mutant in the active site of the protease which is responsible for the cleavage of gag-pol into mature proteins; (b) expression of the mutant in bacterial cells in order to obtain large amounts of uncleaved gag-pol precursor; and (c) purification and characterization of the precursor by activity gel analysis, and peptide mapping.

Immune Response to HIV and Vaccine Development

Results of a six-year prospective study of a cohort of HIV-1-seropositive homosexual men have shown that neutralizing antibodies are correlated with long periods of disease-free status. This observation confirmed and extended our earlier findings of association of neutralizing antibodies with a better clinical outcome in both adult and pediatric patients. Nevertheless, long-term prospective studies of seroconverters will be necessary to establish whether neutralizing antibodies are truly protective, with high titer antibodies correlated with longer time to AIDS onset and prolonged survival. Ongoing studies of the humoral immune responses in HIV-seropositive mothers and their children (in collaboration with Drs. William Blattner and James Goedert, Environmental Epidemiology Branch, NCI) will help establish which responses are protective and associated with healthy, HIV-negative offspring. Results of these studies will influence the composition of future vaccines.

The neutralizing antibody profile obtained in the cohort study showed a plateau level of moderate neutralizing antibody titer which continued for several years. Then, following a marked increase in titer observed over a two-year period, neutralizing activity declined prior to AIDS diagnosis. To better understand the factors contributing to this profile, a molecular epidemiologic study has been initiated. Following extraction of RNA from stored lymphocytes of the same individuals followed for six years, reverse transcription and subsequent polymerase chain reaction will allow analysis of the levels of HIV gene expression during the course of disease progression. In particular, the envelope and regulatory genes are being studied. Results will be analyzed with regard to the immune responses already known for the study subjects, and their overall clinical and immunologic status.

In 1986 we reported the in vitro generation of an HIV-1 variant, obtained by culturing an infectious molecularly cloned virus in the presence of a neutralizing antibody-positive serum. Following extensive molecular analysis of this escape mutant, we subsequently reported that the neutralization resistance was due to a single amino acid substitution at position 582 in the transmembrane protein of the virus. More recent studies using synthetic peptides have shown

that the 582 region is not a neutralization epitope itself. Nevertheless, the variant is resistant to neutralization by 30% of the sera capable of blocking infectivity of the parental virus, indicating that the point mutation in the variant altered a viral characteristic broadly important for HIV infectivity. Current studies are based on the hypothesis that the alanine to threonine substitution at position 582 caused a conformational change in the viral envelope, altering a distant neutralization epitope. Taking advantage of the natural heterogeneity of HIV, selection and analysis of additional escape mutants will allow elucidation of alternate contiguous and conformational epitopes important for viral infectivity and hence for future vaccines.

Using a monoclonal antibody we have mapped an HIV neutralizing epitope to a 24 amino acid region within the viral envelope. Others using alternate techniques have mapped the same site, now recognized as a major type-specific immunodominant epitope. This site is being exploited extensively, with the hope that it will provide at least one component of a subunit vaccine. As this region is very heterogeneous, it is important to know the degree of variability among a number of viral isolates and the range of cross neutralization elicited by specific sequences. We are currently involved in a major collaborative effort (with Drs. D. Zagury of the University Pierre et Marie Currie, Paris, and M. Reitz of the LTCB) analyzing a large number of HIV-1 isolates from Zaire. The cross neutralization studies using sera matched to the individuals from whom the isolates were obtained will tell us the number of neutralization "serotypes" present within a relatively discrete group of individuals at risk of virus infection, and will allow a conclusion concerning whether a mixture of antigens from a range of virus isolates will be necessary in future vaccines. Sequences which elicit the broadest cross neutralizing activity will also be elucidated.

Similar studies aimed at elucidating neutralization epitopes for SIV and HIV-2 are being carried out in collaboration with Drs. G. Franchini of the LTCB and P. Markham of Bionetics Research, Inc., Kensington, MD. Results obtained with these viruses can be applied quickly in primate models and will yield important information for use in HIV-1 research.

Development of a System for Automated Peptide Design and Synthesis

DNA or RNA sequence data can now be converted automatically to overlapping peptides representing the original gene within 24 hours. Specific sequences and sequence analogs or variants can likewise be prepared. This facility will be used to study specificity and function of viral proteins and cellular proteins involved in immune function and in the regulation of viral pathogenesis.

HTLV-I Seroprevalence in the U.S. and Drug Abuser (DA) Populations

Samples were collected by the National Center for Health Statistics using population-based methodology for a National Health Survey, NHANES-II, 1976-1980. Using HIV-1-H9 or HTLV-I-HUT102, 9,499 samples were screened by ELISA. One HIV-1-positive serum from an individual with HIV risk factors was identified. Twenty individuals were seropositive for HTLV-I. None appeared to be HTLV-II-specific. HTLV-I seropositivity was statistically higher in blacks [0.71% (0.23-1.20, 95% CI)] and other minorities [1.03% (0-2.45)] than in whites [0.12% (0.05-0.20)]. HTLV-I seropositivity was statistically higher for people above the age of 45 years -- for white [0.27% (0.09-0.46, 95% C.I.)] and black [1.89%

(0.49-3.4)]. Significant numbers of HTLV-I seroreactive persons exist in regions throughout the U.S. HTLV-I has existed in the U.S. longer than HIV-1. The underlying determinants of positivity in the random population, age and race, were similar to IV DA and other HTLV-I endemic populations. Mode of transmission of HTLV-I, similar to HIV-1 in AIDS risk groups, is unknown for the normal U.S. population. These studies indicate that DAs should be investigated for increased incidence of leukemia and lymphoma related to HTLV-I.

Distribution of HBLV in the Normal Population and Relation to Disease

Sensitive tests for viral antigen and antiviral IgM have been constructed and are currently being used to investigate possible disease relationships with HBLV.

Functional Studies on tat and rev Proteins

We have continued our studies on the structure-function relationship of HIV-1 trans-regulatory genes with an emphasis on virus life cycle. In an early study based on site-directed mutagenesis, we showed that the cysteine residues in the tat trans-activator protein are essential for virus expression. This region of tat is proposed to have a possible role in metal/nucleic acid binding or demineralization. We have recently shown that conserved missense mutations with predicted cysteine to histidine changes within the proposed tetrahedral structure of the nucleic acid binding motif eliminate tat activity and virus expression. In contrast, one cysteine-to-histidine mutation outside the putative tetrahedron had little effect on virus expression. Our studies suggest that although all but one cysteine residue in this domain are important for tat function, the region is unlikely to assume a prototype zinc-finger structure. Furthermore, we suggest that cysteine 31 is not metal-linked in the proposed dimer structure of tat. We have reconstructed our mutant clones in a version that allows a constitutive expression of each mutant under the control of heterologous promoter, i.e., that of cytomegalovirus immediate early gene. These constructs are being tested to elucidate the biochemical properties of the mutant proteins.

In collaboration with Pavlakis et al., Bionetics Research, Inc., we have generated several human established cell lines; each carries a tat- or rev-defective mutant provirus. These cell lines are being utilized as experimental models for HIV latency in vitro. We have found that photosensitization of tat- but not rev-defective cells via ultraviolet (UV) light irradiation rescues transmissible viruses. These revertant viruses display phenotypic characteristics of parental wild-type virus. These studies raise the question of whether natural exposure to UV light may contribute to the pathogenesis of AIDS. We pro-pose that UV radiation of skin might directly or indirectly activate latent virus resident in human epidermal Langerhans cells and/or subepidermal lymphocytes.

The rev trans-regulator protein, though essential for virus replication, appears to play a pivotal role in the balance of HIV-1 gene over- or under-expression. Last year we reported that a rev-defective mutant HIV-1 expresses significantly higher levels of nascent viral RNA than wild-type. We, therefore, postulated that rev, like nef, may play a negative role in virus transcription. Extension of these studies clearly shows that rev exerts both a positive and a negative effect on virus replication, depending on the relative amount of rev supplied in trans. Studies with a reporter gene (CAT) linked to an HIV-1 LTR suggest that

the cis-acting sequence responsive to rev down-regulation is localized to a region in the LTR upstream of the enhancer.

Antisense Oligonucleotides Directed Against HIV-1 Regulatory Genes

In collaboration with Drs. Makoto Matsukura of the Division of Cancer Treatment, NCI, and Eric Wickstrom of the University of South Florida, we have investigated the effects of antisense methylphosphonate oligonucleotides directed against the tat (TAT0, TAT1, TAT3, TAT4, and TAT9) and rev (FWS.1 and FWS.2) messages of HIV-1. Oligonucleotides directed against the TAR site (TAT1) and in the 3' portion noncoding (TAT3) region of tat were found to inhibit virus replication, syncytia formation, and transactivation, and possessed low toxicity for $CD4^+$ lymphocyte cell lines, at levels up to 100 nM. Antisense oligonucleotides directed against the 5' splice-donor site and tat exon 1 initiation site lacked inhibitory activity. Both antisense wild-type and deletion mutant oligonucleotides directed against rev were able to protect SupT1 cells from infection, syncytia formation, and cytotoxic effects after infection or transfection with homologous cloned HIV-1 virus. Further experiments are underway to complete message walking experi-ments with these oligonucleotides and to explore the use of antisense oligo's as tools for the examination of functional sites of HIV-1 regulatory genes.

Mutagenesis of the Envelope per Variable Region (V3) of HIV-1

In collaboration with Steve Petteway and Lucy Ivanoff of Smith, Kline and Beckmann Laboratories, and with James Rusche and Scott Putney of Repligen, Corp., a series of mutants with alterations or deletions of the V3 (HIVAC loop) region, identified as the immunodominant type-specific neutralizing epitope of HIV-1, have been constructed. One of these mutants shows a notably different phenotype from wild-type clones, with slower replication, differential replication in different T-cell lines, and reduced rates of syncytia formation and cytopathogenicity in permissive cell lines. Clones differing at specific residues also show reduced susceptibility to type-specific antisera raised against recombinant peptides (PB1, Sub2) corresponding to the wild-type sequences, complementing and extending previous observations upon the immunogenicity and neutralizing activity of anti-peptide sera made by Drs. Putney, Rusche, Matthews, Bolognesi, and others, as well as our own studies on type-restricted neutralization of molecular clones derived from $HTLV\text{-}III_B$.

Mutants currently constructed include HXB2/10 (pHXB2gpt with the Sall - BamH1 envelope insert of BH10), HXB2/10ala320 (replacement of the highly conserved proline with alanine), HXB2/10thr290 (replacement of the 290 serine residue with threonine), and HXB2/10Δ135 (a mutant with all but 6 residues of the loop deleted). An additional clone, HXB2/10arg313, which changed the BH10 313 serine to arginine, was found to have a deletion and is being reconstructed. The HXB2/10Δ135 mutant was found to be biologically inactive. As mentioned above, the HXB2/10ala320 mutant was found to have markedly altered biological properties, as well as a resistant neutralization phenotype to type-specific sera. Early metabolic labeling experiments suggest that envelope protein processing may be altered in this clone.

Further experiments are planned to examine smaller deletion mutants, express deleted mutants in E. coli and characterize antisera raised against the mutant

envelopes. In addition, replacement of other regions of the loop with sequences from HIV-2 or SIV may yield additional insights into the function of the HIVAC loop.

Transactivation of HIV-1 by HHV-6

We have recently shown that HHV-6 can coinfect with HIV-1 human CD4+ T-cells leading to accelerated cell death, and factors in HHV-6-infected cells stimulate HIV-1 LTR-directed gene expression. We have now examined the mechanism of HIV-1 activation by HHV-6 and localized the cis-acting sequences of HIV-1 LTR responsive to trans-activation. In addition, we have studied the HHV-6-induced trans-activation of other retroviral promoters and found that HHV-6 trans-activates HIV-2 and SIV_{mac}-LTR-linked CAT gene, but not HTLV-I LTR. We demonstrated that increased HIV-1 LTR trans-activation is obtained in HHV-6-infected cells coinfected with HIV-1 or cotransfected with the HIV-1 tat gene. Parallel increases of HIV-1-specific transcripts are seen by in situ hybridization in HHV-6/HIV-1 doubly infected cells as compared to single HIV-1 infection. Similarly, infection by HHV-6 increases the steady state level of HIV-1 LTR mRNA that parallels CAT enzymatic activity, suggesting a transcriptional and/or post-transcriptional activation. Analysis of the sequences necessary for HIV-1 LTR activation by HHV-6 revealed that they are distinct from those required for the tat response and map to a region of the HIV-1 LTR from -103 to -48. Furthermore, the HIV-1 enhancer sequence (-105 to -80) is sufficient to confer HHV-6 inducibility to a heterologous promoter, and nuclear proteins activated or induced by HHV-6 infection specifically bind to the κB motifs of the HIV-1 enhancer region. Since HHV-6 and HIV-1 share similar CD4+ cell tropism, our data suggest that HHV-6 infection may play an important role in the progression of HIV-1 infection to AIDS by increasing HIV-1 gene expression, viral replication and consequently accelerating the cytopathic effect in coinfected CD4+ T-cells both by HIV-1 and by HHV-6 itself.

Cloning and Sequencing of HHV-6

We have developed methods for the growth and purification of HHV-6 and the cognate genomic DNA. The virus can be grown in large quantities (15 liters) in the HSB-2 cell line and concentrated by continuous flow centrifugation using 10-60% sucrose gradients. To purify the viral DNA, the virus is gently lysed and the DNA banded on CsCl gradients. BamH1-restricted HHV-6 genomic DNA yielded 16 fragments of which 11 are now cloned. The sequencing of these fragments which comprise approximately 50% of the HHV-6 genome is nearly completed. The cloning of the additional five high molecular weight fragments is in progress. These are being isolated from preparative agarose gels. Some changes in the restriction pattern of the viral DNA has been noted with passage of the virus. We have regrown the virus from early passage stocks for DNA isolation since the banding patterns were comparable to the prototype virus isolate. Fragments of the viral genome generated with Sal1 and Sst1 are being isolated for cloning. The two enzymes generated 21 and 17 fragments which were easily resolved on agarose gels. These fragments will be cloned and used to create a restriction map of the HHV-6 genome.

We have reported sequence similarity of the HHV-6 genome with the Marek's disease virus (MDV). Most of the hybridization seen under stringent conditions was due to a repeated sequence $(GGGTTA)_n$ shared by the two genomes. The sequence was repeated 26 times in the MDV genome and 60 times in the HHV-6 genome. The

biological significance of these sequences is yet to be determined. The location of the sequences in the MDV IR_S and IR_L junction region is analogous to the "A" sequence in the HSV-1 genome and may function in packaging of the virus or inversion of viral sequences. It is interesting that the repeated sequence is identical to the human telomeric sequence.

Detection of HHV-6 Sequences in Tumors

A survey of hematopoietic tumors by Southern blot analysis showed detectable HHV-6 sequences in three B-lymphoma samples. These were obtained from patients with Burkitt's lymphoma, Sjogren's syndrome and nodular histiocytic lymphoma. A number of Hodgkin's and non-Hodgkin's lymphoma samples and a variety of leukemic specimens of both T- and B-cell types were negative for HHV-6 sequences by Southern blot analysis. An etiological role of HHV-6 in the Burkitt's lymphoma and the Sjogren's lymphoma is unlikely since HHV-6 could not be detected in the majority of Burkitt's samples and some tumor samples from the Sjogren's patient tested negative for HHV-6. Furthermore, in situ hybridization techniques showed that one cell in 10,000 could be detected in the positive Burkitt's lymphoma.

HHV-6 has been determined to be the etiological agent for Roseola Infantum by Yamanishi et al. (Lancet I:1065, 1988). The case for HHV-6 as the etiological agent for some cases of infectious mononucleosis was made by Dr. G. Krueger (University of Cologne, West Germany) in collaboration with members of this laboratory.

The polymerase chain amplification technique was adapted for the detection of low levels of HHV-6 DNA. Peripheral blood DNA of a population of normal blood donors was tested and 32 of 57 tested (56%) were found positive. Correlation to serological positivity was not absolute since some DNA samples from persons who were serologically positive for HHV-6 were negative for HHV-6 sequences. On the other hand, some samples found positive for HHV-6 sequences had been obtained from serologically negative persons. Overall, HHV-6 infection in the population is common and no etiological association with any particular type of cancer has been found.

We found that 52 of 63 (83%) DNA samples from AIDS patients were positive for HHV-6 sequences by PCR technology. Investigations are continuing to determine the role, if any, of HHV-6 in the onset of AIDS.

The usefulness of PCR technology for determining an etiological role of HHV-6 in disease is limited. The prevalence of HHV-6 sequences in the normal population creates difficulties in interpreting data gathered from diseased populations for correlative purposes. Advances have recently been made in using PCR technology to quantitatively estimate the amount of HHV-6 DNA in sample preparations. This remains to be explored. In situ hybridization techniques coupled with histological staining may also be useful in certain cases. This is under development.

Evolutionary Relationship Among Primate Immunodeficiency Retroviruses

In the past year we obtained the entire nucleotide sequence of SIV_{mac} as well as of two HIV-2 viral isolates. These studies allowed us to conclude that the variability among HIV-2s is comparable to the variability observed among African, but not American, HIV-1 isolates. Such findings indicate that HIV-1 and HIV-2

have been in their respective African population for equivalent lengths of time. One important question is whether these retroviruses were transmitted to humans from monkeys. We identified high homology between SIV_{mac} and HIV-2, indicating the possible existence, in west Africa, of a monkey species infected with a virus even more closely related to HIV-2 than SIV_{mac}. Similarly, a simian ancestor of HIV-1 could be identified in monkeys from central Africa where the highest prevalence of HIV-1 seropositive humans has been identified. Our plan is to study the genetic features of SIVs obtained from different geographical areas in Africa. We are presently analyzing five isolates from African green monkeys from Kenya and three isolates from a contiguous region (in collaboration with Drs. P. Markham and D. Zagury).

Characterization of SIV Regulatory Genes

We studied SIV_{mac} regulatory genes and their function. We analyzed the tat, rev, and nef genes which have been cloned as cDNA from SIV_{mac}-infected cells in a mammalian expression vector. The SIV tat gene trans-activates its own LTR as well as HIV-1 LTR, although less efficiently. SIV rev instead is not able to replace the HIV-1 rev when transfected with a HIV-1 rev mutant. The nef gene of HIV-1 has been shown to have a negative regulatory function on viral replication. This effect appears to be mediated by inhibition of transcription from the viral LTR. We examined the ability of the SIV nef gene to down-regulate transcription of a reporter gene linked to the viral LTR and observed that SIV nef protein down-regulates gene expression to a lower extent than HIV-1 nef gene regardless of which LTR we used (HIV-1 or SIV_{mac}). Other properties of nef protein, like GTP bindings and GTPase activity, are under investigation.

Development of a Monkey Animal Model to Study Infection, Pathogenesis and Prevention of HIV Infection

We molecularly cloned two HIV-2 isolates and tested their infectivity in vitro and in vivo. The $HIV\text{-}2_{NIH/z}$ clone is infectious in vitro in fresh and neoplastic human T-cells but does not infect Rhesus macaques in vivo. Conversely, the $HIV\text{-}2_{sbl/isy}$ cloned virus is highly infectious in vivo. We inoculated, in collaboration with Dr. Markham, two juvenile Rhesus macaques one year ago and six more animals six months ago with $HIV\text{-}2_{sbl/isy}$. The first two animals seroconverted approximately two months after the inoculation and showed a decrease in their T-cell count and an inversion of the CD4/CD8 ratio six months after inoculation. Of the other six animals, three seroconverted and we could reisolate virus from the same animals a few months after inoculation. Our goals are: 1) to study the viral genetic determinants for infectivity and pathogenicity. We already constructed mutant viruses in an attempt to increase the infectivity and perhaps the pathogenicity of $HIV\text{-}2_{sbl/isy}$; 2) to immunize the animals using the HIV-2 envelope expressed in various systems and challenge the animals with various doses of viruses; and 3) to try to prevent horizontal and vertical transmission using the viral receptor (CD4) as a molecular decoy or other kind of molecular approach to replace viral replication in vivo.

In Vitro Immunoselection of HIV-1 Variants

One series of studies involved an HIV-1 variant derived from a biologically active molecular clone by transmission and cultivation in the presence of a neutralizing antiserum. The variant was resistant to neutralization by the same antiserum. By a combination of DNA cloning and sequence analysis, construction

of chimeric viruses, and site-specific mutagenesis, we were able to show that a single substitution of threonine for alanine at amino acid position 582 in the transmembrane protein was sufficient to confer the neutralization-resistant phenotype. Other mutants have been constructed which change the primary structure in this region and either change or preserve the local secondary structure. All these mutants remain sensitive to neutralization by the selecting antiserum. In addition, a synthetic peptide representing the wild-type sequence of this region fails to inhibit neutralization. Taken together, the data strongly suggest that the epitope recognized by the neutralizing antibody is conformational and is not simply defined by the primary or secondary structure of the region containing the mutation. This site is of particular interest since it is present in many divergent strains of HIV-1. We are currently analyzing other different neutralization-resistant variants generated by immunoselection.

Genetic Determinants for Macrophage Tropism

We have obtained several molecular clones containing the 3' half of a provirus representing a macrophage tropic HIV-1 (BA-L) and determined the DNA sequence. The organization of the viral genome is identical to T-cell tropic HIV-1 isolates, and the sequence divergence between this isolate and other United States T-cell tropic HIV-1 isolates is no different in kind or degree than that seen among different T-cell tropic HIV-1 isolates. The genetic differences allowing growth in macrophages must, therefore, be rather small. We have constructed different chimeric proviruses by inserting parts of the genome from the macrophage tropic virus into the biologically active clone of the T-cell tropic virus, HTLV-III$_{hxb2}$. These include part or all of the gp120, the amino terminal 250 amino acids of the gp41, and tat and trs. Substitution of the C-terminal 200 amino acids of the gp120 and the N-terminal 150 amino acids of the gp41 of the BA-L virus into HXB2 gives it a limited ability to grow in macrophages, indicating that some but not all of the determinants for macrophage infectivity are contained in this region. Substitution of amino acids 40-300 of the BA-L gp120 into HXB2 give a virus which can only be transmitted by cocultivation, suggesting that in some cases different parts of the envelope proteins of different strains of HIV-1 may not be fully compatible. The construction and characterization of further chimeras is in progress.

Generation and Characterization of HIV-1 Point Mutants

We have made and started to characterize several kinds of mutants of HIV-1. Based on our comparisons of the primary sequence of different HIV-1 isolates, SIV, and various HIV-2 isolates, the 22 cysteine residues of the HIV-1 envelope are completely conserved in all isolates, suggesting that all are functionally important. We have made 14 mutants, each of which have a different cysteine or a pair of cysteines replaced by other amino acids. Seven of nine such mutants lack the ability to infect target cells, confirming the suspected importance of cysteine residues. In general, substitution of cysteine residues in the amino portion of the gp120 result in viruses which are defective in syncytium formation, but competent in envelope glycoprotein production and cleavage and CD4 binding, while substitutions in the carboxyl portion of the gp120 generally result in severe defects in gp160 cleavage and CD4 binding. This suggests that the amino part of gp120 mediates virus-cell fusion, while the carboxyl portion mediates CD4 binding, and that the tertiary structure of the carboxyl portion is necessary for recognition by the protease which cleaves the gp160.

Another series of mutations are being made to characterize the envelope polyprotein cleavage site. Changing the arginine at position 511 to a threonine abolishes envelope cleavage and infectivity. No export of the envelope proteins into the media was observed. The resultant gp160 is able to bind to CD4, but does not have any fusogenic activity. Other mutations are under construction to define the protease recognition site.

A third kind of mutation which is being made and studied is in the coding region for the endonuclease which is important for proviral integration into the host cell genome. Unintegrated DNA is a hallmark of HIV-1 infection, and it is not clear whether or not integration is necessary for infectivity. One of these mutants has a stop codon introduced midway through the integrase coding region. This mutant produces a virus particle which contains RT and all the other viral proteins except the p32 endonuclease. Infection does not, however, seem to be productive, in that virus expression or spread in the target cells has not been noted.

Analysis of PB1 Region of HIV-1 from an Infected African Cohort

Multiple blood samples from a restricted geographical region in Zaire were obtained and virus was isolated. The viral DNA was analyzed by PCR in the region of the PB1 loop, a target of type-specific neutralizing antibodies. Preliminary data show a remarkable conservation of the amino acid sequence of this region. A serologic analysis of this subgenomic region is currently in progress.

Induction of Lymphotoxin Expression by HTLV-I

We analyzed a series of T-cell lines for expression of lymphotoxin and tumor necrosis factor (TNF). These cell lines include uninfected cell lines, cell lines infected by HIV-1, and cell lines established either by in vitro HTLV-I infection or directly from ATL patients. All cell lines infected with HTLV-I constitutively expressed high levels of lymphotoxin RNA, protein, and activity. Many also expressed TNF. In contrast, none of the other cell lines, whether uninfected or infected by HIV-1, expressed detectable levels of either lymphokine. A functional helper T-cell clone expressed no detectable lymphotoxin before infection with HTLV-I; after infection, high levels of expression were noted. One cell line which expressed the transactivator gene, tax, but did not express any structural virus proteins, also expressed high levels of lymphotoxin, suggesting that transactivation of the lymphotoxin promoter is the mechanism of HTLV-I induction of lymphotoxin expression. To investigate this possibility further, we constructed a clone of the lymphotoxin promoter upstream from a reporter gene (CAT). CAT activity was evident after transfection of HTLV-I, but not uninfected or HIV-I-infected T-cell lines, indicating that induction of lymphotoxin indeed is mediated at the level of RNA synthesis. No CAT activity was observed after transfection into Jurkat cells constitutively expressing a transfected tax gene, and these cells also do not express lymphotoxin. Neither they nor untransfected Jurkat cells can be induced to express lymphotoxin. This indicates that tax alone is not sufficient for lymphotoxin induction and that some cellular factor(s) are also required. Deletion mutagenesis of the lymphotoxin gene 5' of the coding sequences showed that the promoter-enhancer region contains both positive and negative regulatory regions. These are being further characterized.

Regulatory Genes and Regulatory Elements of HIV-1 and HIV-2

HIV-1 is etiologically associated with AIDS. More recently, new human retroviruses (termed HIV-2) have been isolated from sick and healthy individuals resident in West Africa. Some of these HIV-2 isolates may be less pathogenic in the natural host. Thus, HIVs may comprise a spectrum of viruses with varying latency and pathogenicity. Our studies explore the premise that the latency and pathogenic potential of these viruses is, at least in part, governed by their genetic structure and that viral gene expression underlies pathogenesis.

HIVs contain several regulatory genes which up-modulate (e.g., tat) down-modulate (e.g., nef, rev) or ensure proper viral gene expression (rev). It is possible that up-modulator genes are weaker and/or down-modulator genes are stronger in less pathogenic HIVs. Thus, we are comparatively analyzing the structural and functional capacities of these genes and of the LTR regulatory elements of HIV-1 and HIV-2, particularly the HIV-2 (strain ST) isolate obtained from a healthy individual. We have determined that, like HIV-1, all of the HIV-2 isolates tested contain a functional tat gene and tat response elements. Detailed mutational analysis has revealed the tat response element of HIV-2 to be more complex than the similar element of HIV-1 and that there are subtle differences in the response of these HIVs to the tat gene products. We have also found that, like HIV-1, HIV-2 expression can be induced by T-cell activation signals and by prototype DNA virus-cytomegalovirus-(CMV)-transactivator (IE-2) gene. Again, there are differences in the details of HIV-1 and HIV-2 induction by T-cell activation and CMV transactivation. Whether these differences are biologically relevant, for example in latency of HIVs, is being ascertained.

Envelope Gene

HIV-2 (strain ST) was isolated from a healthy individual at risk residing in a West African nation (B. Hahn and G. Shaw, University of Alabama, Birmingham, AL). This virus is relatively non-cytopathic in vitro. Transmission and selection of the original HIV-2 (ST) isolate has yielded variants with moderate and high cytopathicity (J. Hoxie). Thus, we now have (non)-weakly cytopathic (ST-N), moderately cytopathic (ST-M) and highly cytopathic (ST-H) variants of HIV-2. Following the premise that envelope protein is relevant to pathogenesis, we are comparatively analyzing the structure and functional properties of the envelope genes of these variants. Thus far, we have obtained biologically active full-length clones of HIV-2 (ST-N) and envelope clones of HIV-2 (ST-N), HIV-2 (ST-M) and HIV-2 (ST-H). The cloning of the full-length genome of HIV-2 (ST-H) is in progress. We have constructed hybrid genomes by exchanging envelope genes, and/or parts thereof, of one variant with the other. These hybrid genomes, along with the parents, will be tested for their capacities for virus replication, syncytia formation, CD4 binding and modulation and cytopathicity.

Virus Neutralizing and Complement Fixing Antibodies

In 1987 we described the absence of complement fixing antibodies (ACC) in 85 human serum samples taken from all stages of disease progression. ACC was also absent from one patient's serum when used against his own virus. We and others found that fresh, normal, human serum was unable to inactivate HIV-1. These findings suggest that serum complement, either directly or through an antibody (IgG was present in all the samples), was unable to contribute to the inactivation of this subfamily of retroviruses.

We recently evaluated the biological relevance of various neutralizing sera from infected humans, chimps and gp120-vaccinated animals given native and recombinant fragments of the envelope. The results suggest that neutralization of HIV-1 occurs through the interaction of Nab and the hypervariable immunodominant epitope (303-321a.a.) on the viral envelope. Also, neutralization can occur after the virion has bound the CD4 receptor (post-binding) and lastly the cell-bound virus is susceptible to Nab for prolonged periods of time (approximately 30 to 45 minutes) Thus, it appears that Nab generated both de novo in HIV-1 infected humans, chimpanzees and gp120-vaccinated animals leads to a very biologically functional Nab. This Nab, however, is highly strain-specific in the vaccinated animals and somewhat broader in neutralization when obtained from infected hosts. These findings, however, only can be applied to lymphocytes infected with HIV-1 as quantitative M/M assays still need to be refined.

Studies previously done in collaboration with Larry Arthur (Program Resources, Inc.) have demonstrated that immunoaffinity purified native envelope gp120 from one particular strain of virus elicits only a homologous, type-specific Nab response. Subsequent studies performed to date to broaden this neutralization reaction include hyperimmunization and piggy-backing another similarly purified unrelated viral envelope to previously immunized host. The results of this study indicate that a paradoxical anamnestic response occurred to the first viral strain used; however, subsequent boosting only gave a Nab against the second viral strain. Thus, individual type-specific Nabs are generated with each HIV-1 variant. Also, hyperimmunization did not broaden the response. Recently, studies in collaboration with Bror Morein and Lennart Akerblom at the National Veterinary Institute, Sweden, formulating the gp120 in an immunostimulatory complex (ISCOM-made out of quil-A glycoside-derivative) have increased the Nab titers tenfold and broadened the Nab response, albeit to a much lower titer, to include a less-related HIV-1 variant.

We served as one of the World Health Organization's international standardization laboratories for the standardization of Nab from various large lots of HIV-1 human serum to be used as reference standards in future HIV research. Also, our laboratory again evaluated two additional panels of Nab from HIV-1 humans using standardization reference lots for the vaccine program in the National Institute of Allergy and Infectious Diseases.

Recently, in a collaborative study with Emilio Emeni of Merck, Sharp and Dohme, West Point, PA, we tested the ability of Nab, derived from a persistently HIV-infected chimpanzee, to protect both a naive chimpanzee and cultured lymphocytes in vitro from challenge with a homologous virus. Additional control antisera included an HIV-1-positive human serum and a Nab monoclonal (0.5B) derived against the homologous challenge virus to be used in the study. Additional controls included IgG purified from both normal humans and chimps. Only the Nab derived from the HIV-1-infected chimpanzee protected the naive chimp from infection. However, in vitro the monoclonal antibody (0.5B), the HIV-positive human serum and the HIV-1-positive chimp serum protected the lymphocytes from infection. Subsequent studies will examine passive immune therapy with this preparation and further elucidate the mechanisms involved in the protection.

HIV-1 Infection of Chimpanzees and a Laboratory Worker

Chimpanzees are infected with as little as 0.2 µl of intravenously administered tissue cultured HIV-1. This represents about one-half to one syncytial-forming

unit of virus and leads to virus reisolation, depending on the concentration of virus in the inoculum, at about two to four weeks. Seroconversion to both gp120 and p24 is found to occur within two weeks of the first virus isolation. Subsequent seroconversion occurs in all the major viral proteins. No abnormalities in T4/T8 ratios have ever been observed in over four years of experimental observation. No viral antigen or infectious virus could be detected in serum samples at any time from any of the animals. Virus isolations are made exclusively from peripheral lymphocytes and are absent from circulating M/M. This was further confirmed by PCR analysis. In addition, the bone marrow was negative for infectious virus. PCR analysis of an HIV-1-infected chimpanzee which died of anesthetic complications 31 days into his infection revealed viral DNA to be present in only his blood lymphocytes. Viral-specific immunohistochemistry revealed a similar distribution of viral antigens, excepting some reactivity in the megakaryocytes of the bone marrow.

In vitro infection of chimp peripheral blood mononuclear cells (C-PBMC) leads to a substantially lower production of cell-free virus and minimal cytopathology compared to identically treated H-PBMC. Purification and infection of the T4 cell subset leads to apparently more viral replication than the C-PBMC experiments; however, no additional cytopathology is detected. In collaboration with Howard Gendelman of WRAI, Silver Spring, MD, purified uninfected chimpanzee M/M appear resistant to productive infection with four strains of human monocytic isolates. Thus, it appears that the HIV-1-infected chimpanzees have serologic and virologic similarities to that of humans; however, other aspects of in vivo viral compartmentalization and controlled viral replication, and lack of cytopathology in vitro are unique to this species.

Serial virus isolation studies from these animals have revealed the presence of neutralization-resistant variants (NRV) in the first isolatable viruses from these animals. These NRVs are serotyped with HIV-1 gp120 antisera specific for the inoculating strain. Additional studies show that the chimpanzees initially make Nab against the inoculating strain and not against the NRVs. Sera taken later from these animals, however, recognizes the early NRVs. All virus libraries have been serotyped, and select animals and virus are undergoing initial amino acid sequence analysis of the immunodominant loop, and secondarily the entire envelope, to map the critical sites associated with the NRV state.

Collaborative studies with Dr. William Blattner involving an accidentally infected laboratory worker have revealed many interesting parallels between HIV-1 infection of the human and chimpanzee. Both behave in a serologically identical manner making antibodies which recognize all the major viral antigen. Virus can be reisolated from PB mononuclear cells. Nab develops more slowly and in lower titer in the human than in the chimpanzee; however, it parallels its biologic behavior exactly. Phenotypic analysis by serotyping has revealed the subsequent serial isolation of increasingly more resistant viruses. Subsequent amino acid sequence analysis done in collaboration with Drs. George Shaw and Beatrice Hahn has revealed concomitant amino acid substitutions in the immunodominant loop apparently responsible for the lack of neutralization seen. These findings suggest that the HIV-1 behaves very similarly in both humans and chimpanzees and further support the need for continued studies with this model. The mechanisms by which these neutralization-resistant viruses are arising -- either de novo or as part of the original inoculum, which now is under a selection phenomena due to Nab following their more rapid replication -- are currently under study in both the human and chimpanzee.

HIV-1 Inhibitory Effect of a Serum Factor and Synthetic Peptides

An extensive survey of the major genera of the animal kingdom has revealed a heat-labile, antiviral serum-based factor(s) present primarily in the rodent and feline family and minimal to no activities in the herbivores, carnivores, and select members of nonhuman primates including the chimpanzee. This activity is dilution-dependent (1:2-1:32) and loses its activity upon heating at 56°C. Further studies have detailed its absolute requirement for Ca++ and its capacity to bind the HIV-1 viral envelope. Furthermore it is capable of preventing infection in vitro when added after virus adsorption. Further studies are underway to elucidate its nature and mechanism of action with hopeful and eventual application as an HIV-1 therapeutic and/or preventive modality.

Benzylated derivatives of peptides corresponding to residues 81 through 92 of the CD4 molecule inhibit fusion between chronically HIV-infected and uninfected CD4-positive cells, and syncytia formation in freshly infected lymphoid cell cultures (Lifson et al., Science 241:712, 1988). To further characterize this activity, recently an in vitro quantitative microassay for acute HIV infection was divided into two kinetic phases corresponding to the two general stages of the viral life cycle: viral infection and transmission of virus and viral protein products through cell contact or release of free virions. To evaluate peptide inhibition at specific stages of the viral life cycle, cultures were treated with peptide either continuously or only during the infection or transmission phases of the assay. CD4 (81-92) peptide derivatives were completely efficacious, at 20-167 μM, to inhibit syncytium formation when present only during the infection phase, the transmission phase, or when present continuously during both phases of the assay. When peptides were present during the infection phase of the assay, inhibition of syncytium formation correlated with decreased p24 expression and lack of secondary infectious cell centers when cells exposed to virus were washed and replated onto fresh uninfected indicator cells.

These data are consistent with complete inhibition of viral infection by peptide, when peptide is present during initial exposure to virus. Unexpectedly, parallel inhibition of syncytium formation decreased p24 levels and inhibition of secondary infectious cell center formation was also seen even when peptides were added as late as 48 hours after inoculation, during the transmission period of the assay. Since viral binding and penetration are believed to be completed well before 48 hours in this assay system, CD4 (81-92) peptide derivatives appear to exert a virustatic effect on cultures already infected with HIV-1, decreasing p24 production, cytopathicity, and cellular infectivity. These results suggest CD4-based antireceptor peptides have antiviral efficacy in mitigating established infection in vitro, and that interactions between the CD4-binding site of the HIV envelope glycoprotein and other cellular or viral components may be important in viral assembly or budding in addition to their established role in viral binding, entry, and HIV-mediated cell fusion.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05534-03 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Monocyte/Macrophages and Accessory Cells in Pathogenesis of HIV-1 Infection

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Popovic	Senior Investigator	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	S. Gartner	Senior Staff Fellow	LTCB NCI
	M. Grofova	Guest Researcher	LTCB NCI
	K. Ohashi	Guest Researcher	LTCB NCI
	A. Minassian	Guest Researcher	LTCB NCI
	F. Michaels	IRTA Fellow	LTCB NCI
	M. Reitz	Research Chemist	LTCB NCI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
5.0	2.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A cell system of peripheral blood-derived monocyte/macrophages (M/M) was successfully employed for human immunodeficiency virus type-1 (HIV-1) isolation in situations where conventional virus isolation failed. We demonstrated that HIV-1 can be recovered from M/M but not from T-cells in several cases of "early" HIV-1 infection or in advanced acquired immune deficiency syndrome (AIDS) cases. The high sensitivity of this system made it possible to recover several HIV-1 isolates termed "low" replicative isolates because of their poor replication in natural targets. Three such isolates have been studied with regard to their expression in M/M. We showed that some cytokines (e.g., colony stimulating factor [CSF]) can accelerate the expression of these "low" replicative isolates in these cells. However, a single and most effective factor for significant increase of HIV-1 replication in M/M infected with these "low" replicative isolates was the number of transmissions of a given viral progeny into recipient cells. Conversion of the "low" replicative human T-lymphotropic virus type-III (HTLV-III-CG-br) isolate into a "high" one occurred within four in vitro passages of this isolate in M/M. The increased virus production was accompanied by the appearance of multinucleated giant cells. Thus, this system of "low" replicative HIV-1 isolates propagated in M/M enables us to study important parameters of virus-host cell interactions which are essential for our understanding of HIV-1 pathogenesis. In addition, biologically active hybrids were generated from two biologically distinct HIV-1 isolates previously characterized by cloning and sequencing of their vDNA. This study permits identification of critical sequences within the HIV-1 genome responsible for significant biological properties.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

M. Popovic	Senior Investigator	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
S. Gartner	Senior Staff Fellow	LTCB NCI
M. Grofova	Guest Researcher	LTCB NCI
K. Ohashi	Guest Researcher	LTCB NCI
A. Minassian	Guest Researcher	LTCB NCI
F. Michaels	IRTA Fellow	LTCB NCI
M. Reitz	Research Chemist	LTCB NCI

Objectives:

Detailed characterization of HIV-1 interactions with natural targets, particularly monocyte/macrophages (M/M); understanding of virus-host cell interactions including adsorption, penetration, persistence and expression of HIV-1 in cells of the mononuclear phagocyte lineage is mandatory for the development of effective therapeutic measures and prevention of development of HIV-1-associated diseases.

Identification of nucleic acid sequences within the HIV-1 genome responsible for efficient replication of HIV-1 isolates in M/M.

Methods Employed:

A variety of virological, immunological, nucleic acid and cell biological techniques are employed. The peripheral blood (PB)-derived M/M culture system, which was developed in our laboratory, is utilized for efficient recovery of HIV-1 from various tissues of virus-infected individuals.

Major Findings:

Persistence and Modulation of HIV-1 Expression in Cells of Mononuclear Phagocyte Lineage

In our previous studies we have shown that the M/M are highly susceptible and permissive host cells for HIV-1. This cell system can be successfully applied for virus isolation in situations when the T-cell system fails. Using PB-derived M/M as targets for HIV-1, the virus was recovered from brain, peripheral blood, bone marrow, lung and skin specimens taken from HIV-1-infected individuals at various stages of disease development. Over 30 HIV-1 isolates have been obtained and most of them characterized by nucleic acid hybridization analysis. It has been successfully demonstrated that in some cases of early infection with the virus (antibody negative, acute infection) or in clinically asymptomatic antibody-positive individuals, HIV-1 could be recovered only from M/M (e.g., $HTLV\text{-}III_B$ infection of a laboratory worker). In addition, the M/M system enabled us to isolate and study the so-called "low" replicative types of HIV-1. Three such isolates have been obtained: one from esophageal tissue of a patient with

acute HIV-1 infection (HTLV-III_{RH-eu}), one from brain (HTLV-III_{cg-br}) and one from thymic tissue (HTLV-III_{cg-thy}) of a pediatric case with full-blown AIDS.

In another study virus has been isolated from PB M/M from sero-negative contacts of HIV-1-infected individuals (e.g., spouses, cohorts) but not T-cells. Nucleic acid analysis of these new isolates has shown them to have unique banding patterns when hybridized to molecular probes of HIV-1.

Studies are continuing to define the level of HIV-1 expression in M/M infected with these "low" replicative isolates as compared to "high" replicative isolates. HIV-1 expression was followed by in situ hybridization and by reverse transcriptase assay. Using the same multiplicity of HIV-1 infection, it was shown that there was at least a 10-fold difference in virus production between "low" and "high" replicative isolates. To delineate which factors can modulate HIV-1 expression, peripheral blood-derived M/M infected with low replicative isolates were exposed to several cytokines. Unlike the case of the model system of HIV-1-infected U-937 neoplastic cells, culture fluids harvested from phytohemagglutinin (PHA)-stimulated T-cells and MLR (mixed lymphocyte reaction) had no effect on HIV-1 expression in M/M infected with "low" replicative HIV-1 isolates. These results suggest that observations obtained from the model system of HIV-1 neoplastic T- or monocytic cell lines may have limited application for in vivo situations of HIV-1-natural host cell interaction. In contrast, using colony stimulating factors (CSF), both the purified CSF as well as the recombinant CSF accelerated the virus expression in M/M which were infected with "low" replicative HIV-1 isolates and cultured in the presence of these lymphokines. In addition, CSF produced by giant cell tumor (GCT) not only accelerated, but actually increased, HIV-1 production in M/M infected with these "low" replicative isolates. However, a single and most effective factor for significant increase of HIV-1 replication in a given M/M infected with "low" replicative HIV-1 isolates was the number of transmissions of a given viral progeny into recipient cells. Conversion of the "low" replicative HTLV-III_{cg-br} isolate into a "high" replicative one occurred within four in vitro passages of the propagated viral progeny in M/M. This increased production of the HTLV-III_{cg-br} isolate was accompanied by the appearance of multinucleated giant cells. Thus, this system of "low" replicative HIV-1 isolates propagated in PB-derived M/M enables us to study important parameters of virus-host cell interactions essential for our understanding of HIV-1 pathogenesis, namely, heterogeneity of HIV-1 isolates and the dynamics (conversion from "low" to "high" production) with respect to cytopathic effect(s) exhibited on host cells.

Identification of the M/M Receptor for HIV-1

The CD4 receptor is the binding site of HIV-1 on the T-cell. Preliminary binding studies of the virus to U-937, a neoplastic promonocytic cell line, have implicated CD4 as the receptor binding site. We are currently studying the receptor binding of HIV-1 in normal PB M/M using monoclonal antibodies against different epitopes of the CD4 receptor to block infection.

Identification of DNA Sequences Within the HIV-1 Genome Responsible for Efficient Infection and Replication of HIV-1 in PB-Derived M/M

In our previous studies we have quantitatively characterized several HIV-1 isolates for their capacity to infect T-cells and M/M. There were significant differences in ED_{50} between different HIV-1 isolates. For instance, $HTLV\text{-}III_{B\,a\text{-}L}$ recovered from and propagated only in M/M productively infected these cells at multiplicities of infection (MOI) of 0.5 to 1 X 10^2 cpm/ml of reverse transcriptase (RT) activity. In contrast, the prototype $HTLV\text{-}III_B$, which readily infects T-cells at these low MOIs, requires an MOI of 5 X 10^5 cpm RT activity/ml to PB-derived M/M. These 1000 to 10000-fold differences between $HTLV\text{-}III_{B\,a\text{-}L}$ and $HTLV\text{-}III_B$ in capacity to infect M/M are sufficient to permit definitive testing of recombinants generated from these two isolates. In collaboration with Dr. M. Reitz, Jr. (Z01CP05538-03 LTCB), a 4.2 Kb Hind-III fragment of $HTLV\text{-}III_{B\,a\text{-}L}$ containing tat, trs, env and a portion of 3' orf has been cloned and sequenced. Nucleic acid sequence analyses have suggested that the 4.2 Kb fragment of the $HTLV\text{-}III_{B\,a\text{-}L}$ (M/M "tropic") isolate does not contain a characteristic portion within the viral genome which would be specifically responsible for efficient infection and replication of the $HTLV\text{-}III_{B\,a\text{-}L}$ isolate in M/M. Data from infection of T-cells and M/M with hybrid viruses generated from the $HTLV\text{-}III_B$ and $HTLV\text{-}III_{B\,a\text{-}L}$ isolates, along with data from sequencing of the vDNA (Hind-III fragment), suggest that minor nucleic acid sequences distributed throughout the whole HIV-1 genome most likely are involved in allowing the efficient replication of an HIV-1 isolate in M/M. Recently, a 6.4 kb EcoRI fragment containing a majority of the envelope region of $HTLV\text{-}III_{B\,A\text{-}L}$ has been cloned and sequenced. Hybrids of this portion of the genome and HTLV-III are being constructed to infect normal T-cells and M/M. Additional information about the genome of M/M tropic isolates is being generated by polymerase chain reaction (PCR). The LTR and a small portion of the GAG region have been sequenced and in the near future constructs will be tested as described previously. Hopefully these experiments will reveal control regions in the HIV-1 genome that will show why certain HIV-1 isolates are T-cell tropic while others are M/M tropic.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05535-03 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Retrovirus Infection, Treatment, Prevention and Etiology of TSP

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. S. Sarin	Research Chemist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	R. Mukhopadhya	Visiting Fellow	LTCB NCI
	T. Ikeuchi	Guest Researcher	LTCB NCI
	F. Lori	Guest Researcher	LTCB NCI
	C. C. Gajdusek	Chief	CNSS NINDS
	C. J. Gibbs	Deputy Chief	CNSS NINDS
	P. R. Johnson	Visiting Scientist	CNSS NINDS

COOPERATING UNITS *(if any)*

George Washington University Medical Center, Washington, DC (A. Goldstein, P. Naylor, R. Schulof); Worcester Fdn. Exp. Biology, Shrewsbury, MA (P. Zamecnik, J. Goodchild); Northwestern University, Chicago, IL (R. Letsinger)

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.5	1.0	2.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

A number of approaches have been examined to identify drugs that can block human immunodeficiency virus type-1 (HIV-1) replication in cell culture. Our studies indicate that a combination of drugs gives a more significant additive and synergistic effect than does single drugs alone, and hence combination chemotherapy may be more useful in the treatment of acquired immunodeficiency syndrome (AIDS). Antisense oligonucleotides containing phosphate, thioate, morpholidate and cholesterol residues have been found to be effective in blocking HIV-1 replication. A combination of oligonucleotides defining different target sites on the HIV-1 genome appears to be synergistic. Both the drugs and antisense oligomers have been found to be effective in inhibiting HIV-1 replication in chronically infected cells as well. Antibodies made against a 30 amino acid HIV-1 p17 synthetic peptide (HGP30) inhibit HIV-1 replication in H9 and Molt-3 cells, and these antibodies appear to be group-specific. One of the HGP30 antisera also inhibited CD4-dependent cell fusion. Pepscan analysis with overlapping nonapeptides derived from the sequence of HIV-1 p17 HGP30 identified the sequence (KE) ALDKIEE (EQ) as the major antibody binding site. HGP30 has been found to be safe and nontoxic in several animal species, and the preparation is ready for vaccine trials in humans. Other synthetic peptides covering the amino acid sequence of p17 are being analyzed for immune response and the development of neutralizing antibodies. A human T-lymphotropic virus type-I (HTLV-I) strain has been isolated from the cerebrospinal fluid (CSF) of a patient with tropical spastic paraparesis (TSP) and this virus isolate appears to be similar to but not identical to HTLV-I from adult T-cell leukemia (ATL). Several HTLV-I isolates have been obtained from TSP patients and the isolate from CSF is being molecularly cloned for further characterization.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

P. S. Sarin	Research Chemist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
R. Mukhopadhya	Visiting Fellow	LTCB NCI
T. Ikeuchi	Guest Researcher	LTCB NCI
F. Lori	Guest Researcher	LTCB NCI
D. C. Gajdusek	Chief	CNSS NINDS
C. J. Gibbs	Deputy Chief	CNSS NINDS
P. R. Johnson	Visiting Scientist	CNSS NINDS

Objectives:

To identify drugs that could interfere with HIV-1 infection and replication and study their mechanism of action. The identification of an active and nontoxic drugs could be very useful in the treatment of AIDS. Advantages of combination therapy with various drugs and immunomodulators are also being explored.

To explore the potential usefulness of synthetic peptides as a vaccine approach for the prevention of AIDS. An HIV-1 p17 synthetic peptide is being evaluated as a vaccine for AIDS. Immune response to a 30 amino acid synthetic peptide (HGP30) is being evaluated in several animal species to determine the type-specific or group-specific antibodies that are produced in these species.

To identify an animal model for AIDS. Studies in several animal species, including chimpanzees, are being carried out.

To examine whether HTLV-I or a variant of HTLV-I is involved in tropical spastic paraparesis (TSP).

Methods Employed:

Standard virological, biochemical and cell biology techniques are utilized in the processing, culture and cocultivation of human and animal cells from peripheral blood, bone marrow and cerebrospinal fluid (CSF). Retrovirus expression in the cell culture is measured by immunofluorescence with monoclonal antibodies against viral antigens, reverse transcriptase assays and electron microscopy. Virus neutralizing antibody and syncytia inhibition assays, as well as assays to examine inhibition of HIV-1 replication in the presence of drugs in freshly infected and chronically infected cells are also utilized.

Major Findings:

Inhibitors of HIV-1 Replication

Several drugs have been examined either alone or in combination for inhibition of HIV-1 replication in cell cultures. Drugs that inhibit virus replication by mechanisms other than reverse transcriptase inhibition and DNA synthesis

chain termination may be useful in the treatment of patients with AIDS and AIDS-related complex (ARC).

D-penicillamine (DPA), which presumably interacts with sulfhydryl group-containing proteins, blocks HIV-1 replication in cell cultures. DPA has been used in the past for the treatment of Wilson's disease, chronic hepatitis and rheumatoid arthritis. This compound acts by inhibiting HIV-1 tat protein expression which is a zinc-containing sulfhydryl protein. A limited clinical trial in AIDS patients is currently in progress.

Amphotericin B methyl ester (AME), a water-soluble derivative of amphotericin B, is a member of the polyene macrolide group of antifungal antibiotics, which interacts with sterols and irreversibly binds to them. It is known to be active against a variety of lipid-enveloped RNA and DNA viruses, several oncogenic retroviruses and different strains of herpesviruses. This antiviral property of AME prompted us to examine its activity against HIV-1, which is also a lipid-enveloped retrovirus. The binding of AME to cholesterol in the membrane of cells causes changes in cell permeability and function, and its binding to sterols of lipid-enveloped viruses causes loss of their infectivity. Both amphotericin B and AME have been found to be potent inhibitors of HIV-1 replication in cell culture (freshly infected and chronically infected) without any toxic side effects. A combination of AME with several other drugs including foscarnet, 3'-azido-3'-deoxythymidine (AZT), 1'-3'-dideoxycytidine (DDC), and dextran sulfate showed both additive and synergistic effects and it appears that combination therapy may be more effective in the treatment of AIDS patients. Both amphotericin B and nystatin as well as their liposomal encapsulated preparations have been found to be effective in inhibiting HIV-1 replication in freshly infected and chronically infected cells.

Avarol and AZT, which inhibit viral replication by different mechanisms, were used to study the role of cytoplasmic factors in independent regulation of host cell and viral gene expression. Both drugs were found to inhibit viral replication and synthesis of virus-encoded protein in a synergistic manner, while at cytostatic drug concentrations, both compounds act antagonistically. ATP-induced transport of viral messengers from isolated nuclei is enhanced by total cytosolic protein from HIV-1-infected cells.

The inhibition of HIV-1 replication by the use of antisense oligonucleotides (synthetic oligonucleotides), which inhibit virus replication by competition hybridization, has been utilized. Since the complete nucleotide sequence of the HIV-1 genome is known, 20 different target sites were selected for the preparation of antisense oligomers. Target sites were selected based on their potential capacity to block recognition functions during viral replication. Antisense oligonucleotides of chain length 20, complementary to sites within or near the sequence repeated at the ends of retrovirus RNA (R region) and to certain splice sites, were most effective. Oligomer derivatives such as thiophos-phates, morpholidates, methylphosphonates and oligomers containing cholesterol residues were also found to be very effective inhibitors of HIV-1 replication in both freshly infected and chronically infected cells. In addition, a combination of several of these oligomers showed both an additive as well as a synergistic effect. Safety and toxicity studies in mice show that these compounds are completely safe and nontoxic. The inhibitory activity of

antisense oligomers containing methylphosphonate residues was found to be dependent on both chain length and the number of phosphonate residues. Introduction of 18 phosphonate groups in an oligomer of chain length 20 significantly increased HIV inhibitory activity relative to the parent oligonucleotide, whereas incorporation of five methylphosphonate residues showed little or no increase in HIV inhibition capacity.

Use of Synthetic Peptides as Vaccines for AIDS

Several approaches to the development of an AIDS vaccine are being pursued in different laboratories. They include the envelope glycoprotein, vaccinia vector, anti-idiotypes, and synthetic peptides as sources for an AIDS vaccine. We have explored the potential usefulness of synthetic peptides as candidate AIDS vaccines, especially a peptide generated from the p17 sequence of HIV-1. The p17 protein was recently found, by electron microscopy using immunogold labeling techniques, by Gelderblom et al. (Max Planck Institute, Berlin, FRG; unpublished results) to be associated with the envelope glycoproteins of HIV-1. Similar conclusions have been drawn by computer modeling techniques. This observation suggests that HIV-1 p17 epitope may be exposed on the surface in the virions. Hence, gag gene products are important in any consideration of a potential vaccine for AIDS. In addition, because of the genetic variability in the gp120 region of different HIV-1 isolates, which show as much as a 20% divergence in the amino acid sequence of gp120, it is important to look for other approaches to the development of an AIDS vaccine.

A 30 amino acid peptide analog of HIV-1 p17 (termed HGP30) and other analogs spanning the HIV-1 p17 amino acid sequence have been synthesized that cross-react in the HIV-1 p17 radioimmunoassays (RIA) and can be identified by HIV-1 p17 monoclonal antibodies by enzyme-linked immunosorbent assays (ELISA) and Western blotting. Antibodies prepared against HGP30 inhibit HIV-1 replication in cell culture. These studies indicate that HIV-1 p17 may be important in the cell-mediated immunity and that these antibodies may be protective against HIV-1 infection. Pepscan analysis with overlapping non-peptides derived from the sequence of HIV-1 p17 HGP30 identified the sequence (KE) ALDKIEE (EQ) as the major antibody binding site. Sera of 9% of AIDS patients (7/76) and 18% of HIV-1 seropositive healthy homosexuals (40/223) were positive for HGP30 antibodies. Decline in HIV-1 p17 antibodies has been shown to be related to disease progression in both children and adults suggesting that HIV-1 p17 antibodies may be protective. A limited clinical trial with HGP30 is currently in progress in England.

Tropical Spastic Paraparesis (TSP) and Neurological Dysfunction

Adult T-cell leukemia (ATL) and TSP are diseases that are prevalent in Jamaica and the Caribbean, areas which are endemic for HTLV-I. HTLV-I antibodies have been identified in a large number of patients with TSP. TSP is a neurological syndrome in which patients progressively develop difficulty in walking, stiffness of legs and back pain. A similar disease called HTLV-I-associated myelopathy (HAM) has recently been described in Japan. We have isolated HTLV-I strains from the CSF and peripheral blood lymphocytes of TSP patients from Jamaica and Colombia. The HTLV-I strain from CSF of a TSP patient shows differences in the prototype HTLV-I isolate of ATL patients, by restriction enzyme mapping, suggesting that the TSP HTLV-I strain is similar to but not identical to the

prototype HTLV-I isolate. A genomic library of this HTLV-I strain has been obtained and is being subcloned for further characterization. Several cell lines developed from TSP patients have been characterized phenotypically by using a panel of monoclonal antibodies to lymphoid cell surface markers, such as CD3, CD4, CD8, CD25, and HLA-DR.

Three of the TSP cell lines and two ATL cell lines have been adapted to total serum-free medium and the supernatants are being used to determine the secretion of some major interleukins and soluble IL-2 receptor. The mRNA from these cell lines will also be examined for the presence of mRNA for various interleukins and the IL-2 receptor.

Analysis of HIV-1 Reverse Transcriptase Maturation Process

Identification and characterization of HIV-1, HIV-2, and SIV gag-pol precursors: HIV synthesizes a gag-pol fusion protein which is later cleaved to give mature gag and pol proteins. The identification and analysis of such a precursor has been limited until now by the low amount of protein that can be recovered from infected cells or mature virions. With the activity gel analysis we have shown the presence of 165 KDa (HIV-1) and 170 KDa (HIV-2, SIV) catalytically active bands that may represent the entire product of the gag-pol gene. Studies are in progress to obtain large quantities of the precursor for further characterization by (a) construction of an HIV-1 mutant in the active site of the protease which is responsible for the cleavage of gag-pol into mature proteins; (b) expression of the mutant in bacterial cells in order to obtain large amounts of uncleaved gag-pol precursor; and (c) purification and characterization of the precursor by activity gel analysis, and peptide mapping.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05536-03 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immune Response to HIV: Neutralizing Antibody and Vaccine Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Robert-Guroff	Research Biologist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	M. S. Reitz, Jr.	Research Chemist	LTCB NCI
	G. Franchini	Visiting Scientist	LTCB NCI
	W. A. Blattner	Chief, Family Studies Section	EEB NCI
	J. Goedert	Medical Officer	EEB NCI

COOPERATING UNITS *(if any)*

Repligen Corp., Cambridge, MA (S. Putney); Bionetics Research Inc., Rockville, MD (P. Markham); University Pierre et Marie Currie, Paris, France (D. Zagury)

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In studying immune responses to human immunodeficiency virus (HIV) infection, we have focused on neutralizing antibodies which we first detected in 1985. Initial studies in infected children and adults showed that neutralizing antibodies are associated with a better clinical outcome. A six-year prospective study found that such antibodies were correlated with long periods during which HIV-infected homosexual men maintained good health. Declining or low antibody titers indicated poor prognosis. Future studies of HIV seroconverters will clarify whether neutralizing antibodies retard disease onset or prolong survival. Studies of HIV transmission from mothers to children will help elucidate immune responses important for protection against infection. The effect of HIV envelope heterogeneity on the elicitation and function of neutralizing antibodies is also being pursued. While a major type-specific neutralizing epitope has been localized to a 24 amino acid region of the HIV envelope, investigation of an immune-selected HIV variant has revealed that a substitution of threonine for alanine at position 582 in the viral transmembrane protein caused resistance to neutralization by the selecting serum. Thus, additional contiguous or conformational epitopes may be important for neutralizing antibody development. Analysis of other escape mutants will further define these regions. Cross-neutralization studies of HIV isolates of a known envelope sequence will elucidate which sequences elicit antibody of the broadest specificity and may, therefore, be important components of future vaccines. Similar studies of immune responses following SIV and HIV-2 infection are being carried out to elucidate neutralization epitopes in these viruses and allow testing of vaccine approaches in animal model systems.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

M. Robert-Guroff	Research Biologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
M. S. Reitz, Jr.	Research Chemist	LTCB NCI
G. Franchini	Visiting Scientist	LTCB NCI
W. A. Blattner	Chief, Family Studies Section	EEB NCI
J. Goedert	Medical Officer	EEB NCI

Objectives:

The objectives of this project are to learn what natural immune surveillance mechanisms operate in HIV-1-infected individuals, whether such natural responses can be manipulated to enhance protection against virus infection or disease progression, and how to construct vaccine preparations to result in such protective immune responses. As retroviral neutralizing antibodies are known to result in protection against a viral challenge in animal systems, we have initially focused on these antibodies. The specific goals of the present project are:

1. to determine to what extent HIV-1 neutralizing antibodies are protective or influence disease progression;

2. to determine what influence the known heterogeneity of the viral envelope has on elicitation and function of HIV-1 neutralizing antibodies;

3. to identify regions of the virus envelope important for viral infectivity and neutralization;

4. to determine the efficacy of various envelope preparations, including recombinant constructions, subunit fragments, and anti-idiotypic antibodies, as vaccine materials; and

5. to probe immune responses and modes of transmission of other human and primate retroviruses in order to apply knowledge gained to investigations on HIV, while further elucidating mechanisms of pathogenesis and protection in other retroviral systems.

Methods Employed:

The method for assaying sera for HIV-1 neutralizing antibodies has been described by M. Robert-Guroff *et al*. (Nature 316:72, 1985). Long-term prospective studies of neutralizing antibodies in HIV-1-infected individuals were carried out on a cohort of 34 homosexual men, prospectively followed since 1982. These same individuals form the study population for the analysis of gene expression (*env*, *tat*, *rev*, *nef*) during disease progression.

The methodology for immune selection of HIV variants in vitro has been described (Robert-Guroff et al., J. Immunol. 137:3306, 1986) as have procedures for their molecular analysis (Reitz et al., Cell 54:57, 1988). Sera chosen to supply selective pressure in the HIV-1 system include high titered neutralizing patient sera, and animal sera hyperimmunized with peptides representing known neutralizing epitopes (supplied by Dr. Putney). Either patient or natural animal sera select for escape mutants in the HIV-2 and SIV systems. Parental viruses used in these experiments include stock HTLV-IIIB, HTLV-IIIMn, and HTLV-IIIRF; the infectious molecular clone HXB2D; and HXB2D into which portions of other viral envelopes have been inserted.

Stable stocks of infectious preparations of all viruses used are prepared and titered for use in cross-neutralization studies. Routine serologic assays for antibodies to HIV-1 and other retroviruses are carried out using the ELISA technique, Western blotting, radioimmunoprecipitation and immune fluorescent assays. Reverse transcription of RNA extracted from frozen lymphocytes of patients is carried out using the enzyme from avian myeloblastosis virus. The polymerase chain reaction is carried out following procedures outlined by the kit manufacturer (Perkin-Elmer). Reaction products are analyzed on agarose, acrylamide, or nusieve gels depending on their predicted size.

Major Findings:

Results of a six-year prospective study of a cohort of HIV-1-seropositive homosexual men have shown that neutralizing antibodies are correlated with long periods of disease-free status. This observation confirmed and extended our earlier findings of association of neutralizing antibodies with a better clinical outcome in both adult and pediatric patients. Nevertheless, long-term prospective studies of seroconverters will be necessary to establish whether neutralizing antibodies are truly protective, with high titer antibodies correlated with longer time to AIDS onset and prolonged survival. Ongoing studies of the humoral immune responses in HIV-seropositive mothers and their children (in collaboration with Drs. W. Blattner and J. Goedert) will help establish which responses are protective and associated with healthy, HIV-negative offspring. Results of these studies will influence the composition of future vaccines.

The neutralizing antibody profile obtained in the cohort study showed a plateau level of moderate neutralizing antibody titer which continued for several years. Then, following a marked increase in titer observed over a two-year period, neutralizing activity declined prior to AIDS diagnosis. To better understand the factors contributing to this profile, a molecular epidemiologic study has been initiated. Following extraction of RNA from stored lymphocytes of the same individuals followed for six years, reverse transcription and subsequent polymerase chain reaction will allow analysis of the levels of HIV gene expression during the course of disease progression. In particular, the envelope and regulatory genes are being studied. Results will be analyzed with regard to the immune responses already known for the study subjects, and their overall clinical and immunologic status.

In 1986 we reported the in vitro generation of an HIV-1 variant, obtained by culturing an infectious molecularly cloned virus in the presence of a neutralizing antibody-positive serum. Following extensive molecular analysis of

this escape mutant, we subsequently reported that the neutralization resistance was due to a single amino acid substitution at position 582 in the transmembrane protein of the virus. More recent studies using synthetic peptide have shown that the 582 region is not a neutralization epitope itself. Nevertheless, the variant is resistant to neutralization by 30% of the sera capable of blocking infectivity of the parental virus, indicating that the point mutation in the variant altered a viral characteristic broadly important for HIV infectivity. Current studies are based on the hypothesis that the alanine to threonine substitution at position 582 caused a conformational change in the viral envelope, altering a distant neutralization epitope. Taking advantage of the natural heterogeneity of HIV, selection and analysis of additional escape mutants will allow elucidation of alternate contiguous and conformational epitopes important for viral infectivity and hence for future vaccines.

Using a monoclonal antibody we have mapped an HIV neutralizing epitope to a 24 amino acid region within the viral envelope. Others using alternate techniques have mapped the same site, now recognized as a major type-specific immunodominant epitope. This site is being exploited extensively, with the hope that it will provide at least one component of a subunit vaccine. As this region is very heterogeneous, it is important to know the degree of variability among a number of viral isolates and the range of cross neutralization elicited by specific sequences. We are currently involved in a major collaborative effort (with Drs. D. Zagury and M. Reitz) analyzing a large number of HIV-1 isolates from Zaire. The cross neutralization studies using sera matched to the individuals from whom the isolates were obtained will tell us the number of neutralization "serotypes" present within a relatively discrete group of individuals at risk of virus infection, and will allow a conclusion concerning whether a mixture of antigens from a range of virus isolates will be necessary in future vaccines. Sequences which elicit the broadest cross neutralizing activity will also be elucidated.

Similar studies aimed at elucidating neutralization epitopes for SIV and HIV-2 are being carried out in collaboration with Drs. G. Franchini and P. Markham. Results obtained with these viruses can be applied quickly in primate models and will yield important information for use in HIV-1 research.

Publications:

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Patents:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05537-03 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunopathogenesis of Human RNA and DNA Viruses

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	W. C. Saxinger	Research Microbiologist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	F. Wong-Staal	Chief, Molecular Biology Section	LTCB NCI
	P. Levine	Medical Officer	EEB NCI

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

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NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.0	1.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Viral pathogenesis: Work on the chimpanzee human immunodeficiency virus (HIV) model has suggested new directions for approaches to intervention. Findings are that infection appears to progress by discrete stages which may be variably immunoregulated and that cofactors or cellular immunity, or target cell selection may be fundamental. Also, in vitro tests of B- and T-cell immunosuppression by viral proteins and fragments produced by molecular biological techniques have been successful in the preliminary phase. Detailed characterization of mechanisms of immunosuppression are in progress.

U.S. human T-lymphotropic virus type-I (HTLV-I) prevalence and relation to disease: A retrospective random sampling of the U.S. population (HANES-II) and a retrospective geographic drug abuser population have been tested for HTLV-I antibody. Analysis in progress will indicate frequency of infection and its rate of change in these populations. The range of clinical manifestations of HTLV-I infection will be monitored in high risk groups such as IV drug abusers.

Human B-lymphotropic virus (HBLV) prevalence and relation to disease: Enzyme-linked immunosorbent assay (ELISA) tests have been successfully developed. Prevalence studies, geographically and epidemiologically oriented, have shown that immunoglobulin (IgG) reactivity to HBLV in normal adults is common (>80%) and that exposure to the virus takes place frequently within the first year after birth. Selected disease groups will be tested for the progression of viral infection by monitoring anti-HBLV IgM and direct tests for viral antigen.

Molecular structure analysis of proteins related to HIV/HTLV pathogenesis, related growth and regulatory factors, and prevention of disease: Aspects of cell-receptor binding, humoral and cellular immune response, vaccine design will be studied by combined approaches of peptide synthesis, and molecular modelling.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

W. C. Saxinger	Research Microbiologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
F. Wong-Staal	Chief, Molecular Biology Section	LTCB NCI
P. Levine	Medical Officer	EEB NCI

Objectives:

Molecular Definition of Viral Protein Functions

To study functional aspects of viral protein interactions and specificity by the combined approaches of peptide synthesis and molecular modelling.

Modulation of T- and B-Cell Function by Viral Proteins

Characterization of stimulatory and suppressive effects on human lymphocytes produced by HTLV/HIV proteins. Comparison of various HTLV variants with respect to these characteristics in support of studies of mechanisms important in the pathogenesis and spread of HTLV/HIV.

Identification of New Virus Clusters in Humans and Primates

Identification of the environmental distribution of HTLV or of variant viruses related to HTLV in support of studies of mechanisms important in the pathogenesis and spread of HTLV/HIV.

Immunological and Structural Characterization of HBLV

Development of a screening test for antibodies to HBLV in various human populations for studies of disease association. Identification of immunogenic viral proteins and characterization of human humoral response. Translate open reading frames of HBLV sequence into synthetic peptides to identify important viral proteins.

Characterization of Disease Associations with HBLV

Progression of viral antigen with a newly developed antigen capture test and IgG/IgM status will be monitored in candidate lymphoproliferative and immunosuppressive disease states.

Methods Employed:

A variety of immunochemical, immunological, and microbiological techniques are used. Antigen-antibody reactions are measured by standard and in-house developed ELISA, radioimmunoassay (RIA), Western blot, and a variety of other immunoprecipitation techniques. Purification of cellular and viral proteins is accomplished by a variety of chromatographic (gravity and high performance liquid

chromatography [HPLC]), electrophoretic, and centrifugation techniques. Large-scale epidemiologic data analysis is performed using a lab-based personal computer coupled with an IBM mainframe system for demographic data entry and storage of immunological test data. Assays for cellular immunity and modulation are performed by standard in vitro tests for hematopoietic cell function by incorporation of radiolabelled, growth or specific plaque assays. Peptide syntheses are performed by standard (9-fluorenylmethyoxycarbonyl amino group protection [FMOC]) chemical procedures integrated with automation devices developed within the LTCB.

Major Findings:

Development of a System for Automated Peptide Design and Synthesis

DNA or RNA sequence data can now be converted automatically to overlapping peptides representing the original gene within 24 hours. Specific sequences and sequence analogs or variants can likewise be prepared. This facility will be used to study specificity and function of viral proteins and cellular proteins involved in immune function and in the regulation of viral pathogenesis.

HTLV-I Seroprevalence in the U.S. and Drug Abuser (DA) Populations

Samples were collected by the National Center for Health Statistics using population-based methodology for a National Health Survey, NHANES-II, 1976-1980. Using HIV-1-H9 or HTLV-I-HUT102, 9,499 samples were screened by ELISA. One HIV-1-positive serum from an individual with HIV risk factors was identified. Twenty individuals were seropositive for HTLV-I. None appeared to be HTLV-II-specific. HTLV-I seropositivity was statistically higher in blacks [0.71% (0.23-1.20, 95% CI)] and other minorities [1.03% (0-2.45)] than in whites [0.12% (0.05-0.20)]. HTLV-I seropositivity was statistically higher for people above the age of 45 years -- for white [0.27% (0.09-0.46, 95% C.I.)] and black [1.89% (0.49-3.4)]. Significant numbers of HTLV-I sero-reactive persons exist in regions throughout the U.S. HTLV-I has existed in the U.S. longer than HIV-1. The underlying determinants of positivity in the random population, age and race, were similar to IV DA and other HTLV-I endemic populations. Mode of transmission of HTLV-I, similar to HIV-1 in AIDS risk groups, is unknown for the normal U.S. population. These studies indicate that DAs should be investigated for increased incidence of leukemia and lymphoma related to HTLV-I.

Distribution of HBLV in the Normal Population and Relation to Disease

Sensitive tests for viral antigen and anti-viral IgM have been constructed and are currently being used to investigate possible disease relationships with HBLV.

Publications:

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Patents:

Gallo RC, Salahuddin SZ, Saxinger WC, Ablashi DV. Canadian Patent (Pending): Testing for the Human B-lymphotropic Virus (HBLV).

Gallo RC, Salahuddin SZ, Saxinger WC, Ablashi DV. US Patent (Pending): Testing for HBLV.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05538-03 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Structure and Function of HIV Genome

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Reitz	Research Chemist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	M. Robert-Guroff	Research Biologist	LTCB NCI
	M. Popovic	Research Biologist	LTCB NCI
	S. Gartner	Senior Staff Fellow	LTCB NCI
	E. Tschachler	Visiting Scientist	LTCB NCI
	H.-G. Guo	Visiting Scientist	LTCB NCI
	C. Wilson	Microbiologist	LTCB NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

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SECTION

Molecular Genetics of Hematopoietic Cells

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Conservation of certain areas of the genomes of the HIV-1, HIV-2 and SIV are helping to identify those which are functionally critical. Based in part on these analyses, mutants of HIV-1 have been made and are being studied. These include mutations at the envelope polyprotein cleavage site (which in preliminary studies result in loss of infectivity), within the integrase gene (which also resulted in loss of infectivity), and at various cysteine residues in the envelope proteins (some of which resulted in loss of infectivity). Another project has involved the selection, in vitro by neutralizing antisera, of HIV-1 variants which resist neutralization by the selecting antiserum. DNA sequence analyses, construction of viral chimeras, and site-specific mutagenesis of one such variant showed resistance was due to a single amino acid substitution in the transmembrane protein. This has obvious implications for vaccine design. Other variants are currently under study. A third project involves analysis of HIV-1 isolates from a cohort of infected individuals in Zaire. The region of the env gene coding for the PB1 region, which is a frequent target of type-specific neutralizing activity in hyperimmune sera, will be amplified by the polymerase chain reaction (PCR) technique. These will be compared and the comparative immune response of the autologous sera will be analyzed. This should give insight into the structural basis for recognition by type-specific neutralizing antibodies, as well as preliminary data on the kind and degree of heterogeneity which should be built into any candidate vaccines. A fourth project is to identify the genetic determinants which give some strains of HIV-1 the ability to grow on macrophages as well as on T-cells. Several DNA clones of a macrophage tropic HIV-1 were obtained and completely sequenced. These were used to generate and analyze a series of viral chimeras containing different genomic regions derived from the macrophage tropic parent.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

M. Reitz	Research Chemist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
M. Robert-Guroff	Research Biologist	LTCB NCI
M. Popovic	Research Biologist	LTCB NCI
S. Gartner	Senior Staff Fellow	LTCB NCI
E. Tschachler	Visiting Scientist	LTCB NCI
M.-G. Guo	Visiting Scientist	LTCB NCI
C. Wilson	Microbiologist	LTCB NCI

Objectives:

This work is an effort to understand how structural aspects of the genes of HIV relate to viral biology, including different steps in replication, cell tropism, and interaction with the host immune system. Much of this work concerns the envelope gene and its relationship to the above properties.

Methods Employed:

Standard techniques of molecular biology and virology are being used to pursue these investigations.

Major Findings:

In Vitro Immunoselection of HIV-1 Variants

One series of studies involved an HIV-1 variant derived from a biologically active molecular clone by transmission and cultivation in the presence of a neutralizing antiserum. The variant was resistant to neutralization by the same antiserum. By a combination of DNA cloning and sequence analysis, construction of chimeric viruses, and site-specific mutagenesis, we were able to show that a single substitution of threonine for alanine at amino acid position 582 in the transmembrane protein was sufficient to confer the neutralization-resistant phenotype. Other mutants have been constructed which change the primary structure in this region and either change or preserve the local secondary structure. All these mutants remain sensitive to neutralization by the selecting antiserum. In addition, a synthetic peptide representing the wild-type sequence of this region fails to inhibit neutralization. Taken together, the data strongly suggest that the epitope recognized by the neutralizing antibody is conformational and is not simply defined by the primary or secondary structure of the region containing the mutation. This site is of particular interest since it is present in many divergent strains of HIV-1. We are currently analyzing other different neutralization-resistant variants generated by immunoselection.

Genetic Determinants for Macrophage Tropism

We have obtained several molecular clones containing the 3' half of a provirus representing a macrophage tropic HIV-1 (BA-L) and determined the DNA sequence.

The organization of the viral genome is identical to T-cell tropic HIV-1 isolates, and the sequence divergence between this isolate and other United States T-cell tropic HIV-1 isolates is no different in kind or degree than that seen among different T-cell tropic HIV-1 isolates. The genetic differences allowing growth in macrophages must, therefore, be rather small. We have constructed different chimeric proviruses by inserting parts of the genome from the macrophage tropic virus into the biologically active clone of the T-cell tropic virus, HTLV-III_{hxb2}. These include part or all of the gp120, the amino terminal 250 amino acids of the gp41, and tat and trs. Substitution of the C-terminal 200 amino acids of the gp120 and the N-terminal 150 amino acids of the gp41 of the BA-L virus into HXB2 gives it a limited ability to grow in macrophages, indicating that some but not all of the determinants for macrophage infectivity are contained in this region. Substitution of amino acids 40-300 of the BA-L gp120 into HXB2 give a virus which can only be transmitted by cocultivation, suggesting that in some cases different parts of the envelope proteins of different strains of HIV-1 may not be fully compatible. The construction and characterization of further chimeras is in progress.

Generation and Characterization of HIV-1 Point Mutants

We have made and started to characterize several kinds of mutants of HIV-1. Based on our comparisons of the primary sequence of different HIV-1 isolates, SIV, and various HIV-2 isolates, the 22 cysteine residues of the HIV-1 envelope are completely conserved in all isolates, suggesting that all are functionally important. We have made 14 mutants, each of which have a different cysteine or a pair of cysteines replaced by other amino acids. Seven of nine such mutants lack the ability to infect target cells, confirming the suspected importance of cysteine residues. In general, substitution of cysteine residues in the amino portion of the gp120 result in viruses which are defective in syncytium formation, but competent in envelope glycoprotein production and cleavage and CD4 binding, while substitutions in the carboxyl portion of the gp120 generally result in severe defects in gp160 cleavage and CD4 binding. This suggests that the amino part of gp120 mediates virus-cell fusion, while the carboxyl portion mediates CD4 binding, and that the tertiary structure of the carboxyl portion is necessary for recognition by the protease which cleaves the gp160.

Another series of mutations are being made to characterize the envelope polyprotein cleavage site. Changing the arginine at position 511 to a threonine abolishes envelope cleavage and infectivity. No export of the envelope proteins into the media was observed. The resultant gp160 is able to bind to CD4, but does not have any fusogenic activity. Other mutations are under construction to define the protease recognition site.

A third kind of mutation which is being made and studied is in the coding region for the endonuclease which is important for proviral integration into the host cell genome. Unintegrated DNA is a hallmark of HIV-1 infection, and it is not clear whether or not integration is necessary for infectivity. One of these mutants has a stop codon introduced midway through the integrase coding region. This mutant produces a virus particle which contains reverse transcriptase and all the other viral proteins except the p32 endonuclease. Infection does not, however, seem to be productive, in that virus expression or spread in the target cells has not been noted.

Analysis of PB1 Region of HIV-1 from an Infected African Cohort

Multiple blood samples from a restricted geographical region in Zaire were obtained and virus was isolated. The viral DNA was analyzed by PCR in the region of the PB1 loop, a target of type-specific neutralizing antibodies. Preliminary data show a remarkable conservation of the amino acid sequence of this region. A serologic analysis of this subgenomic region is currently in progress.

Publications:

Colombini S, Arya SK, Reitz MS, Jagodzinski L, Beaver B, Wong-Staal F. Structure of SIV regulatory genes. Proc Natl Acad Sci USA (In Press).

Gurgo C, Guo HG, Franchini G, Aldovini A, Farrell K, Wong-Staal F, Gallo RC, Reitz MS Jr. Envelope sequences of two new United States HIV-1 isolates. Virology 1988;164:531-6.

Reitz MS, Wilson C, Naugle C, Gallo RC, Robert-Guroff M. Generation of a neutralization-resistant variant of HIV-1 is due to selection for a point mutation in the envelope gene. Cell 1988;54:57-63.

Reitz MS, Wilson C, Naugle C, Gallo RC, Robert-Guroff M. Immunoselection of a neutralization-resistant variant of HIV-1. In: Compans R, Helenius A, Oldstone M, eds. Proceedings of UCLA symposium on cell biology of virus entry, replication and pathogenesis. New York: Alan R Liss, 1988;551-3.

Wilson C, Reitz MS, Okayama H, Eiden MV. Formation of infectious hybrid virions with Gibbon ape leukemia virus and human T-cell leukemia virus retroviral envelope glycoproteins and the gag and pol proteins of Moloney murine leukemia virus. J Virol 1989;63:2374-8.

Yourno J, Josephs SF, Reitz M, Zagury D, Wong-Staal F, Gallo RC. Nucleotide sequence analysis of the env gene of a new Zairean isolate of HIV-1. AIDS Res Hum Retroviruses 1988;4:165-73.

Zagury JF, Franchini G, Reitz MS Jr, Collalti E, Starcich B, Hall L, Fargnoli K, Jagodzinski L, Guo HG, Zagury D, Wong-Staal F, Gallo RC. The genetic variability between HIV-2 isolates is comparable to the variability among HIV-1. Proc Natl Acad Sci USA 1988;85:5941-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05539-03 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Determinants of the Latency and Pathogenicity of Human Retroviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	S. K. Arya	Research Biologist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	M. Kaplan	Guest Researcher	LTCB NCI

COOPERATING UNITS *(if any)*

University of Alabama, Birmingham, AL (B. Hahn, G. Shaw); University of Pennsylvania, Philadelphia, PA (J. Hoxie); Bionetics Research Laboratories, Kensington, MD (V. S. Kalyanaraman)

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human immunodeficiency viruses (HIVs) may comprise a spectrum of human retroviruses with varying latency and pathogenicity. These properties may be governed, in part, by their genetic structure. This project aims to comparatively analyze the structure and function of the genomes of the highly pathogenic (HIV-1, HIV-2 [NIHZ]) and weakly pathogenic (HIV-2[ST]) HIVs with particular focus on their regulatory genes (tat, nef) and the envelope genes. Potentially relevant to the latency and pathogenicity is the property of these viruses to be activated by non-HIV factors, such as T-cell activation and heterologous transactivation, e.g., by concomitant infection with some oncogenic DNA viruses. We and others have shown that all strains of HIV-1 and HIV-2 contain functional tat gene and tat response elements. However, there are subtle differences in the HIV-1 versus HIV-2 tat-mediated transactivation. Similarly, the expression of both HIV-1 and HIV-2 can be induced by T-cell activation and cytomegalovirus (CMV) trans-activator (IE-2) gene. This activation is largely due to the transcriptional activation involving transcript initiation and elongation. Interestingly, the regulatory elements that respond to T-cell activation in HIV-2 are distinct from similar elements in HIV-1. Whether these differences, as in tat response, are biologically relevant to the latency, remains to be ascertained. The role of the envelope gene in pathogenesis is being evaluated by constructing hybrid genomes where envelopes of highly pathogenic and weakly pathogenic HIV-2s have been exchanged. These hybrid genomes are being introduced into susceptible cells by DNA transfection and evaluated for virus production, syncytia formation, CD4 binding and modulation, and cytopathicity.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

S. K. Arya	Research Biologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
M. Kaplan	Guest Researcher	LTCB NCI

Objectives:

The major objective of this project is to evaluate the determinants of the latency and pathogenicity of HIV and to utilize this information for developing therapeutic modalities. An additional and related objective is to dissect the mechanisms of the regulation of HIV gene expression.

Methods Employed:

Molecular cloning in expression vectors, DNA sequencing, DNA-mediated transfection analysis, transcriptional and translational analyses, reverse transcription assays, CD4 binding and modulation, syncytia formation and other standard methodologies of gene cloning and recombinant DNA technology.

Major Findings:

HIV-1 is etiologically associated with acquired immune deficiency syndrome (AIDS). More recently, new human retroviruses (termed HIV-2) have been isolated from sick and healthy individuals resident in West Africa. Some of these HIV-2 isolates may be less pathogenic in the natural host. Thus, HIVs may comprise a spectrum of viruses with varying latency and pathogenicity. Our studies explore the premise that the latency and pathogenic potential of these viruses is governed, at least in part, by their genetic structure and that viral gene expression underlies pathogenesis.

Regulatory Genes and Regulatory Elements of HIV-1 and HIV-2

HIVs contain several regulatory genes which up-modulate (e.g., tat), down-modulate (e.g., nef, rev), or ensure proper viral gene expression (rev). It is possible that up-modulator genes are weaker and/or down-modulator genes are stronger in less pathogenic HIVs. Thus, we are comparatively analyzing the structural and functional capacities of these genes and of the long terminal repeat (LTR) regulatory elements of HIV-1 and HIV-2, particularly the HIV-2 (strain ST) isolate obtained from a healthy individual. We have determined that, like HIV-1, all of the HIV-2 isolates tested contain a functional tat gene and tat response elements. Detailed mutational analysis has revealed the tat response element of HIV-2 to be more complex than the similar element of HIV-1 and that there are subtle differences in the response of these HIVs to the tat gene products. We have also found that, like HIV-1, HIV-2 expression can be induced by T-cell activation signals and by prototype DNA virus-CMV-transactivator (IE-2) gene. Again, there are differences in the details of HIV-1

and HIV-2 induction by T-cell activation and CMV transactivation. Whether these differences are biologically relevant, for example in latency of HIVs, is being ascertained.

Envelope Gene

HIV-2 (strain ST) was isolated from a healthy individual at risk residing in a West African nation (B. Hahn and G. Shaw). This virus is relatively non-cytopathic in vitro. Transmission and selection of the original HIV-2 (ST) isolate has yielded variants with moderate and high cytopathicity (J. Hoxie). Thus, we now have (non)-weakly cytopathic (ST-N), moderately cytopathic (ST-M) and highly cytopathic (ST-H) variants of HIV-2. Following the premise that envelope protein is relevant to pathogenesis, we are comparatively analyzing the structure and functional properties of the envelope genes of these variants. Thus far, we have obtained biologically active full-length clones of HIV-2 (ST-N) and envelope clones of HIV-2 (ST-N), HIV-2 (ST-M) and HIV-2 (ST-H). The cloning of the full-length genome of HIV-2 (ST-H) is in progress. We have constructed hybrid genomes by exchanging envelope genes, and/or parts thereof, of one variant with the other. These hybrid genomes, along with the parents, will be tested for their capacities for virus replication, syncytia formation, CD4 binding and modulation and cytopathicity.

Publications:

Arya SK. Human immunodeficiency retroviruses: activation and differential transactivation of gene expression. AIDS Res Hum Retroviruses 1988;4:175-86.

Arya SK, Gallo RC. Human immunodeficiency virus type 2 long terminal repeat: analysis of regulatory elements. Proc Natl Acad Sci USA 1988;85:9753-7.

Colombini S, Arya SK, Reitz MS, Jagodzinski L, Beaver B, Wong-Staal F. Structure of SIV regulatory genes. Proc Natl Acad Sci USA (In Press).

Zagury JF, Franchini G, Reitz MS Jr, Collalti E, Hall L, Fargnoli KA, Jagodzinski L, Guo HG, Laure F, Zagury D, Arya SK, Josephs SF, Wong-Staal F, Gallo RC. The genetic variability between HIV-2 isolates is comparable to the variability among HIV-1. Proc Natl Acad Sci USA 1988;85:5941-5.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05560-02 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Induction of Lymphotoxin Expression by HTLV-I Infection

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	M. Reitz	Research Chemist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	E. Tschachler	Visiting Scientist	LTCB NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Molecular Genetics of Hematopoietic Cells

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Adult T-cell leukemia, which is caused by human T-lymphotropic virus type-I (HTLV-I), is frequently characterized by severe hypercalcemia and lytic bone lesions. The lymphokine lymphotoxin, synthesized by T-cells, has among its activities the ability to activate osteoclasts. We, therefore, examined HTLV-I-infected T-cells for evidence of lymphotoxin expression. High levels of constitutive lymphotoxin expression were noted in all HTLV-I-infected cells, but not in uninfected T-cells or those infected by human immunodeficiency virus (HIV-1). HTLV-I-mediated lymphotoxin expression appears to be dependent on the viral *tax* gene, and to act at the level of RNA transcription. The effect of *tax* is likely indirect, operating through interaction with several cellular factors.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

M. Reitz	Research Chemist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
E. Tschachler	Visiting Scientist	LTCB NCI

Objectives:

The major objective of this work is to understand the molecular mechanism of hypercalcemia in adult T-cell leukemia (ATL). We also wish to identify cellular factors important in lymphokine expression which are also important in virus expression.

Methods Employed:

Standard molecular biological recombinant DNA methods are being used in these investigations.

Major Findings:

Induction of Lymphotoxin Expression by HTLV-I

We analyzed a series of T-cell lines for expression of lymphotoxin and tumor necrosis factor (TNF). These cell lines include uninfected cell lines, cell lines infected by HIV-1, and cell lines established either by in vitro HTLV-I infection or directly from ATL patients. All cell lines infected with HTLV-I constitutively expressed high levels of lymphotoxin RNA, protein, and activity. Many also expressed TNF. In contrast, none of the other cell lines, whether uninfected or infected by HIV-1, expressed detectable levels of either lymphokine. A functional helper T-cell clone expressed no detectable lymphotoxin before infection with HTLV-I; after infection, high levels of expression were noted. One cell line which expressed the transactivator gene, tax, but did not express any structural virus proteins, also expressed high levels of lymphotoxin, suggesting that transactivation of the lymphotoxin promoter is the mechanism of HTLV-I induction of lymphotoxin expression. To investigate this possibility further, we constructed a clone of the lymphotoxin promoter upstream from a reporter gene (CAT). CAT activity was evident after transfection of HTLV-I, but not uninfected or HIV-I-infected T-cell lines, indicating that induction of lymphotoxin indeed is mediated at the level of RNA synthesis. No CAT activity was observed after transfection into Jurkat cells constitutively expressing a transfected tax gene, and these cells also do not express lymphotoxin. Neither they nor untransfected Jurkat cells can be induced to express lymphotoxin. This indicates that tax alone is not sufficient for lymphotoxin induction and that some cellular factor(s) are also required. Deletion mutagenesis of the lymphotoxin gene 5' of the coding sequences showed that the promoter-enhancer region contains both positive and negative regulatory regions. These are being further characterized.

Publications:

Tschachler E, Robert-Guroff M, Gallo RC, Reitz MS. Human T-lymphotropic virus I-infected cells constitutively express lymphotoxin in vitro. Blood 1989;73:194-201.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05614-01 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Immunobiology of HIV-1: Neutralizing Antibody and Vaccine Development

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. L. Nara	Expert	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	N. M. Dunlop	Microbiologist	LTCB NCI
	M. J. Merges	Microbiologist	LTCB NCI
	C. M. Poore	Biologic Lab. Tech.	LTCB NCI

COOPERATING UNITS *(if any)*

PRI, Frederick, MD (L. Arthur, S. Pyle, S. Conley); National Veterinary Institute, Uppsala, Sweden (B. Morein); Repligen Corp., Cambridge, MA (S. Putney); Merck, Sharp & Dohme, West Point, PA (E. Emeni)

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.50	0.25	1.25

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Understanding and characterizing the protective and functional humoral antibody response is critical in the design of any prototypic viral vaccine. Studying these responses in naturally or accidentally infected human and animal models (such as the chimpanzee) capable of replicating the human agent, are pivotal. Analysis of anti-HIV antibody produced in response to infection was found to be devoid of complement activating activity. The neutralizing antibody (Nab) produced in both humans and chimpanzees is initially type-specific and broadens at a later time to include multiple strains. The Nab was found to be capable of neutralizing the virus sometime after it had bound the CD4 receptor and before it had penetrated the cell membrane. Additionally in these studies, we discovered that susceptibility to Nab was prolonged on the cell membrane and post CD4 binding (90% of the virus could be neutralized for 45 to 60 minutes). Thus, the high affinity receptor interaction between virus and lymphoid cell can be overcome. Vaccine studies utilizing native and recombinant envelope proteins and its fragments (the immunodominant hypervariable loop - 303-320a.a.) also provoke the production of a similar type of Nab; however, the neutralizing response is restricted to the immunizing strain. Manipulation with alternate novel adjuvants such as ISCOMS have increased titers and broadened, to some degree, the Nab response. Lastly, we demonstrated that Nab produced in experimentally infected chimpanzees was protective against in vitro and in vivo infection and thus serves as a bench mark for our vaccine studies involving Nab.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

P. L. Nara	Expert	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
N. M. Dunlop	Microbiologist	LTCB NCI
M. J. Merges	Microbiologist	LTCB NCI
C. M. Poore	Biologic Lab. Tech.	LTCB NCI

Objectives:

The objectives of this project are to evaluate the humoral immune responses in HIV-1 experimentally infected chimpanzees, infected humans, as well as, subunit, viral, envelope-based vaccines formulated with various conventional and novel adjuvants. There is a precedence for retroviral-based envelope vaccines to protect animals from leukemogenesis through the generation of various functional antiviral antibodies, that is, neutralizing and complement fixing, and thus warrant a thorough investigation as a necessary component of an AIDS vaccine. The specific goals of the parent project were:

1. to evaluate the various characteristics of the antibody produced under these conditions, i.e., neutralizing antibody (Nab) and complement fixing antibody;

2. to evaluate the biological relevancy of Nab in infected humans, chimpanzees and experimentally immunized animals by detailed kinetic analysis for evaluation of the rate and stage at which it works, as well as the breadth of its neutralizing activity (Is it isolate-specific or capable of inactivating many unrelated variants?);

3. to evaluate means to broaden the limited specificity of Nab through various immunization protocols in lab animals;

4. to participate in a national and international program to standardize neutralization assays for HIV-1 and HIV-2; and

5. to test the protective effects of Nab derived from a monoclonal HIV-1-positive human polyclonal and an HIV-1-infected chimpanzee polyclonal both in vitro and in vivo.

Methods Employed:

The method for assaying the various sera for HIV-1 neutralizing antibodies has been previously described by Nara et al. (AIDS Res Hum Retroviruses 3:283, 1987; Nature 331:469, 1988). Detailed kinetic analyses were done as described by Nara (Cold Spring Harbor, Vaccines 89, p. 137). Sera were tested against seven HIV-1 variants: IIIB and its three clones, HX-10, HXB2, HXB3, RF, MN and CC. The viral stocks were optimized for infectivity/defectivity ratios, clarified and, when possible, cryopreserved and subsequently titered.

Immunoaffinity-purified HIV-1 viral envelopes were obtained as previously described by Robey et al. (Science 228:593, 1985) and Pyle et al. (AIDS Res Hum Retroviruses 3:387, 1987).

Routine serologic analyses were carried out on all serum samples using radio-immunopreciptation, enzymne-liked immunosorbent assay (ELISA), competitive radioimmunoassay, Western blotting and immunofluorescence.

In collaboration with Scott Putney of Repligen Corporation, polyclonal antibody was derived specifically against the immunodominant loop of various HIV-1 isolates as described.

Major Findings:

In 1987 we described the absence of complement fixing antibodies (ACC) in 85 human serum samples taken from all stages of disease progression. ACC was also absent from one patient's serum when used against his own virus. We and others found that fresh, normal, human serum was unable to inactivate HIV-1. These findings suggest that serum complement, either directly or through an antibody (IgG was present in all the samples), was unable to contribute to the inactivation of this subfamily of retroviruses.

We recently evaluated the biological relevance of various neutralizing sera from infected humans, chimps and gp120-vaccinated animals given native and recombinant fragments of the envelope. The results suggest that neutralization of HIV-1 occurs through the interaction of Nab and the hypervariable immunodominant epitope (303-321a.a.) on the viral envelope. Also, neutralization can occur after the virion has bound the CD4 receptor (post-binding) and lastly the cell-bound virus is susceptible to Nab for prolonged periods of time (approximately 30 to 45 min.) Thus, it appears that Nab generated both de novo in HIV-1 infected humans, chimpanzees and gp120-vaccinated animals leads to a very biologically functional Nab. This Nab, however, is highly strain-specific in the vaccinated animals and somewhat broader in neutralization when obtained from infected hosts. These findings, however, only can be applied to lymphocytes infected with HIV-1 as quantitative monocyte/macrophage assays still need to be refined.

Studies previously done in collaboration with Larry Arthur of Program Resources, Inc. have demonstrated that immunoaffinity-purified native envelope gp120 from one particular strain of virus elicits only a homologous, type-specific Nab response. Subsequent studies performed to date to broaden this neutralization reaction include hyperimmunization and piggy-backing another similarly purified unrelated viral envelope to a previously immunized host. The results of this study indicate that a paradoxical anamnestic response occurred to the first viral strain used; however, subsequent boosting only gave a Nab against the second viral strain. Thus, individual type-specific Nabs are generated with each HIV-1 variant. Also, hyperimmunization did not broaden the response. Recently, studies in collaboration with Bror Morein and Lennart Akerblom at the National Veterinary Institute, Sweden, formulating the gp120 in an immunostimulatory complex (ISCOM-made out of quil-A glycoside-derivative) have increased the Nab titers tenfold and broadened the Nab response, albeit to a much lower titer, to include a less-related HIV-1 variant.

We served as one of the World Health Organization's international standardization laboratories for the standardization of Nab from various large lots of HIV-1 human serum to be used as reference standards in future HIV research. Also, our laboratory again evaluated two additional panels of Nab from HIV-1 humans using standardization reference lots for the vaccine program in the National Institute for Allergy and Infectious Diseases.

Recently, in a collaborative study with Emilio Emeni, we tested the ability of Nab, derived from a persistently HIV-infected chimpanzee, to protect both a naive chimpanzee and cultured lymphocytes in vitro from challenge with a homologous virus. Additional control antisera included an HIV-1-positive human serum and a Nab monoclonal (0.5B) derived against the homologous challenge virus to be used in the study. Additional controls included IgG purified from both normal humans and chimps. Only the Nab derived from the HIV-1-infected chimpanzee protected the naive chimp from infection. However, in vitro the monoclonal antibody (0.5B), the HIV-positive human serum and the HIV-1-positive chimp serum protected the lymphocytes from infection. Subsequent studies will examine passive immune therapy with this preparation and further elucidate the mechanisms involved in the protection.

Publications:

Arthur LO, Pyle SW, Nara PL, Bess JW Jr, Kelliher JC, Gilden RV, Robey WG, Fischinger PJ. Preparation and evaluation of an HIV-1 gp120 prototype vaccine. In: Ginsberg H, Brown F, Lerner RA, Channock RM, eds. Vaccines 88, 1988;277-82.

Nara PL. HIV-1 neutralization: evidence for rapid, binding/postbinding neutralization from infected humans, chimpanzees and gp120-vaccinated animals. In: Lerner RA, Ginsberg H, Channock RM, Brown F, eds. Vaccines 89, 1989;127-44.

Nara PL, Fischinger PJ. Quantitative infectivity microassay for HIV-1 and -2. Nature 1988;331:469-70.

Nara PL, Robey WG, Pyle SW, Hatch WC, Dunlop NM, Bess JW Jr, Kelliher JC, Arthur LO, Fischinger PJ. Purified envelope glycoproteins from human immunodeficiency virus type 1 induce individual type-specific neutralizing antibodies. J Virol 1988;62:2622-8.

Pyle S, Bess J, Arthur L, Morein B, Lerche N, Kelliher J, Nara P. Primate immunizations with HIV-1 gp120 immunostimulatory complexes. In: Lerner RA, Ginsberg H, Channock RM, Brown F, eds. Vaccines 89, 1989;201-6.

Zagury D, Bernard J, Cheynier R, Desportes I, Leonard R, Fouchard M, Reveil B, Hele DI, Lurhuma Z, Mbayo K, Wane J, Saluan J, Goussard B, Dechazal L, Burny A, Nara PL, Gallo RC. A group-specific anamnestic immune reaction against HIV-1 induced by determinants of the viral envelope glycoprotein, gp160: a candidate vaccine prototype against AIDS. Nature 1988;332:728-31.

Patents:

Nara PL, Dunlop NM, Fischinger PJ, Hatch WC. (Pending): Quantitative, Syncytium-forming Microassay for the Detection of Human Immunodeficiency Virus Neutralization (pending on the CEM-SS assay/cell line).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05615-01 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biology of HIV-1 In Chimpanzees and an Accidentally Infected Lab Worker

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. L. Nara	Expert	LTCB NCI
Others:	N. M. Dunlop	Microbiologist	LTCB NCI
	M. J. Merges	Microbiologist	LTCB NCI
	C. M. Poore	Biologic Lab. Tech.	LTCB NCI
	J. Ward	Chief, Tumor Pathol. & Pathogenesis Section	LCC NCI

COOPERATING UNITS *(if any)*

Acad Med Ctr, Amsterdam, The Netherlands (J. Goudsmit); SUNY Hlth Sci Ctr, Syracuse, NY (G. Ehrlich); WRAI, Silver Spring, MD (H. Gendelman); PRI, Frederick, MD (L. Arthur, D. Waters, S. Conley)

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.25	0.25	1.0

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Animal models of human disease are important comparative tools in research. The development and characterization of an HIV-1 animal model such as the chimpanzee, for persistent viral infection serves just such a purpose. Chimpanzees (pan-troglodytes) are uniquely susceptible to intravenous infection; as little as 0.2 µl or 0.5 syncytial-forming units of tissue culture-derived virus is capable of causing an infection. Viruses can be readily reisolated from their peripheral blood mononuclear cells (C-PBMCs) for long periods (months to years). Seroconversion occurs within two weeks of initial virus isolation and antibodies made, recognize all major viral proteins. No cell-free virus can be detected in the plasma and no immunologic or cytologic abnormality has been characterized for over four years. Virus appears to be contained in the circulating peripheral lymphocyte pool only. Comparative in vitro infections of human and C-PBMCs leads to reduced virus replication and minimal virus-associated cytopathology as compared to HIV-1-infected human PBMCs.

Comparative studies in an accidentally infected lab worker, who received a similar virus strain as our chimpanzee studies, suggest similar serologic and virologic post-infectious events. The infected human responds as the chimpanzee does with a type-specific neutralizing antibody followed by a more broadly reactive response. The human, however, has an overall slower and lower titered response. Also, neutralization-resistant variants are reisolated over a 28-month period and show point mutations in the immunodominant neutralization epitope.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

P. L. Nara	Expert	LTCB NCI
N. M. Dunlop	Microbiologist	LTCB NCI
M. J. Merges	Microbiologist	LTCB NCI
C. M. Poore	Biologic Lab. Tech.	LTCB NCI
J. Ward	Chief, Tumor Pathol. & Pathogenesis Section	LCC NCI

Objectives:

The objectives of this project are to investigate all aspects of HIV-1 infection in man and the nearest living relative of man, the chimpanzee (pantroglodytes). As only gibbons and chimpanzees have been shown to be persistently infectable by the virus, understanding the host responses, resultant changes in the virus, as well as standardizing an infectivity model to evaluate future prototypic, anti-HIV vaccines are all critical and responsible goals in such a special and limited research resource.

1. Perform minimal infective dose titrations with standardized, pre-titered cryopreserved stocks of HIV-1.

2. Characterize the model, immunologically, virologically, clinically and serologically, to evaluate reproducibility and its comparative biological relatedness to a laboratory-acquired, persistent accidental homologous strain, HIV infection of a human.

3. Evaluate sequential virus isolations from chimpanzees by serotyping and molecular analysis. Study the evolving Nab response to parental virus and subsequent reisolated variants. Map hypermutable site within the viral envelope to better understand those epitopes responsible for both neutralization and escape from neutralization.

4. Study the distribution of the virus in the chimpanzee as well as comparative in vitro studies with HIV-1-infected chimp and human lymphocytes and macrophages.

Methods Employed:

Virus for challenge stock was grown in H9 cells, clarified, cryopreserved and titered in human and chimp primary lymphocytes as well as transformed human T-cell lines. Virus isolation was done with PHA-stimulated target and normal human donor lymphocytes. Positive virus isolations were determined by reverse transcriptase and p24 antigen capture. Serotyping was performed with nine quantitative microtiter HIV-1 infectivity assay as described by Nara *et al.* (AIDS Res Hum Retroviruses 3:283, 1987).

Polymerase chain reaction on various samples were done in collaboration with Garth Ehrlich as described by Abbot *et al.* (J Infec Dis 158:1158, 1988) using *gag*- and *env*-specific primers. Monocytes and macrophages were derived from Ficoll-hypaque gradients and subsequently stimulated with a granulocyte/macrophage colony stimulation factor(s) as described by Nara *et al.*

(J Med Primatol, in press) and Gendelman et al. (J Exp Med 167:1428, 1988). Viral envelopes from HIV-1-infected chimpanzees were PCR amplified, sequenced, and serum tested for specific peptide reactivity by pep scan in collaboration with Jaap Goudsmit. Viral envelopes from the lab worker were similarly amplified, cloned, and sequenced by George Shaw (University of Alabama, Birmingham, AL). Viral-specific immunohistochemistry was performed as previously described by Ward et al. (Am J Pathol 127:199, 1987).

Major Findings:

Chimpanzees are infected with as little as 0.2 µl of intravenously administered tissue-cultured HIV-1. This represents about one-half to one syncytial-forming unit of virus and leads to virus reisolation, depending on the concentration of virus in the inoculum, at about two to four weeks. Seroconversion to both gp120 and p24 is found to occur within two weeks of the first virus isolation. Subsequent seroconversion occurs in all the major viral proteins. No abnormalities in T4/T8 ratios have ever been observed in over four years of experimental observation. No viral antigen or infectious virus could be detected in serum samples at any time from any of the animals. Virus isolations are made exclusively from peripheral lymphocytes and are absent from circulating monocyte/macrophages. This was further confirmed by PCR analysis. In addition, the bone marrow was negative for infectious virus. PCR analysis of a HIV-1 infected chimpanzee which died of anesthetic complications 31 days into his infection revealed viral DNA to be present in only his blood lymphocytes. Viral-specific immunohistochemistry revealed a similar distribution of viral antigens, excepting some reactivity in the megakaryocytes of the bone marrow.

In vitro infection of chimp peripheral blood mononuclear cells (C-PBMC) leads to a substantially lower production of cell-free virus and minimal cytopathology compared to identically treated H-PBMC. Purification and infection of the T4 cell subset leads to apparently more viral replication than the C-PBMC experiments; however, no additional cytopathology is detected. In collaboration with Howard Gendelman purified uninfected chimpanzee monocyte/macrophages appear resistant to productive infection with four strains of human monocytic isolates. Thus, it appears that the HIV-1-infected chimpanzees have serologic and virologic similarities to that of humans; however, other aspects of in vivo viral compartmentalization and controlled viral replication, and lack of cytopathology in vitro are unique to this species.

Serial virus isolation studies from these animals have revealed the presence of neutralization-resistant variants (NRV) in the first isolatable viruses from these animals. These NRVs are serotyped with HIV-1 gp120 antisera specific for the inoculating strain. Additional studies show that the chimpanzees initially make Nab against the inoculating strain and not against the NRVs. Sera taken later from these animals, however, recognizes the early NRVs. All virus libraries have been serotyped, and select animals and virus are undergoing initial amino acid sequence analysis of the immunodominant loop, and secondarily the entire envelope, to map the critical sites associated with the NRV state.

Collaborative studies with Dr. William Blattner (Environmental Epidemiology Branch, NCI) involving an accidentally infected lab worker have revealed many interesting parallels between HIV-1 infection of the human and chimpanzee. Both behave in a serologically identical manner making antibodies which recognize all the major viral antigen. Virus can be reisolated from peripheral blood

mononuclear cells. Nab develops more slowly and in lower titer in the human than in the chimpanzee; however, it parallels its biologic behavior exactly. Phenotypic analysis by serotyping has revealed the subsequent serial isolation of increasingly more resistant viruses. Subsequent amino acid sequence analysis done in collaboration with George Shaw and Beatrice Hahn has revealed concomitant amino acid substitutions in the immunodominant loop apparently responsible for the lack of neutralization seen. These findings suggest that the HIV-1 behaves very similarly in both humans and chimpanzees and further support the need for continued studies with this model. The mechanisms by which these neutralization-resistant viruses are arising --either de novo or as part of the original inoculum, which now is under a selection phenomena due to Nab following their more rapid replication -- are currently under study in both the human and chimpanzee.

Publications:

Nara PL. HIV-1 neutralization: evidence for rapid, binding/postbinding neutralization from infected humans, chimpanzees and gp120-vaccinated animals. In: Lerner RA, Ginsberg H, Channock RM, Brown F, eds. Vaccines 89: modern approaches to new vaccines including prevention of AIDS. New York: Cold Spring Harbor Laboratory, 1989;137-44.

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Zagury D, Bernard J, Cheynier R, Desportes I, Leonard R, Fouchard M, Reveil B, Hele DI, Lurhuma Z, Mbayo K, Wane J, Salua J, Goussard B, Dechazal L, Burny A, Nara PL, Gallo RC. A group-specific anamnestic immune reaction against HIV-1 induced by determinants of the viral envelope glycoprotein, gp160: a candidate vaccine prototype against AIDS. Nature 1988;332:728-31.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05616-01 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Anti-HIV Factors in Animal Sera and CD4 Anti-receptor Therapy for HIV-1

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. L. Nara	Expert	LTCB NCI
Others:	N. M. Dunlop	Microbiologist	LTCB NCI
	L. E. Eiden	Unit Head	LCB NIMH
	D. Raush	Staff Fellow	LCB NIMH
	M. Padgett	Chemist	LCB NIMH
	C. M. Poore	Biologic Lab. Tech.	LTCB NCI
	S. Hosoi	Visiting Fellow	LIB NCI

COOPERATING UNITS *(if any)*

Genelabs Inc., Redwood City, CA (J. Lifson, K. Hwang); FDA, Bethesda, MD (B. Fraser); USUHS, Bethesda, MD (T. Borsos)

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The absence of serum complement antiviral activity seen in lentiviral diseases of humans, sheep and goats and the contributing factor(s) associated with its genus-species restriction were the major initiatives in this project. A systematic analysis for anti-HIV viral inactivating activity of sera from various members of the animal kingdom was performed. Sera from the rodent and feline families were potent inhibitors of HIV infection in vitro. The serum factor(s) were heat-labile, 56°C for 30 minutes and completely dependent on Ca++. The sera were capable of inactivating, in a dose-dependent fashion, all strains of HIV-1 and HIV-2 tested. Further characterization is ongoing in attempts to understand its mechanism of action for application to the human AIDS condition.

Successful pharmacological interventive therapeutic modalities have been derived from the study of receptor-based agonist/antagonist cogeners. A novel therapeutic approach to a receptor-based agonist-viral strategy was examined by synthetic peptide synthesis. Sequential, overlapping peptides were constructed for the CD4 receptor of the human T-cell and a known receptor for HIV. A benzylated, derivatized by-product with sequence specificity and anti-viral activity was observed in the 81-92aa binding domain of CD4. This material is capable of preventing cell-free viral infection and fusion of HIV-1-infected cells at 20-160 μM concentrations small enough to cross cell membrane, and in preliminary studies appears to have a virostatic effect on HIV-1-infected cells. Further structure-function activity studies are ongoing to enhance the potency and understand the mechanism of viral inactivation as well as map-critical binding regions of the viral envelope.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

P. L. Nara	Expert	LTCB NCI
N. M. Dunlop	Microbiologist	LTCB NCI
L. E. Eiden	Unit Head	LCB NIMH
D. Raush	Staff Fellow	LCB NIMH
M. Padgett	Chemist	LCB NIMH
C. M. Poore	Biologic Lab. Tech.	LTCB NCI
S. Hosoi	Visiting Fellow	LIB NCI

Objectives:

The objectives of this project were to investigate some of the genus-species restricted basis of infectivity for the lentiviruses. Human immunodeficiency viruses (HIV-1 and HIV-2) as well as other animal lentiviruses are known to be resistant to the antiviral effects of homologous species complement. We systematically investigated the animal kingdom for its ability to mediate a complement-like antiviral effect. 1) Survey all major genera for serum-based, antiviral, "complement-like" activity. 2) Test the breadth of this antiviral activity with multiple divergent HIV-1 and HIV-2 isolates as well as determine the stage of inactivation. 3) Elucidate and purify both the mechanism and protein(s) responsible for this activity. 4) Investigate the factors involved in the absence or refractoriness of human serum to mediate this activity.

Synthetically derivatize small (25 aa) segments of the HIV-1 T-cell receptor, the CD4 molecule, and test for their inherent ability to block viral infection, cell fusion, and viral replication in vitro. Their small size was designed to maximize the antiviral effect and minimize the molecular size to afford their penetration across biological membranes such as cell and blood brain barriers (BBB). 1) Determine the smallest critical receptor site antagonist for antiviral effect in the CD4 molecule, capable of crossing the BBB and not eliciting antibody against itself. 2) Evaluate the various in vitro conditions and stages of the viral infection/replication cycle, including cell fusion, that are affected by these molecules. 3) Determine the breadth of antiviral activity on numerous strains of HIV-1, HIV-2 and SIV.

Methods Employed:

Antiviral activities were tested in the quantitative HIV-1, HIV-2 infectivity microassay as described by Nara *et al.* (AIDS Res Hum Retroviruses 3:283, 1987; Nature 331:469, 1988). C_1 and complement hemolytic titers and analysis of the divalent cations were done with a component analysis approach using various standard chelating agents as previously described by Borsos *et al.* (Science 150:505, 1965).

The peptide mixture CD4 (81-92)BZL, referred to as "peak 7" and indicated as TYIC(S-benzyl)EVEDZKEE, was programmed for synthesis on an Applied Biosystems 430A peptide synthesizer using PAM resin, alpha-N-tBoc symmetric anhydrides of all amino acids except glutamine (HOBT ester), and conventional R-protecting groups, with the exception of N-tBoc-S-benzyl-cysteine, chosen to minimize

S-deprotection during cleavage. Twenty-seven individual syntheses of S-benzyl-CD4(81-92) resulted in peptide mixtures with nominal activities in the fusion inhibition assay, prior to chromatographic purification, of 238 ± 99 μM (mean ± standard deviation, n = 26 individual tBOC syntheses performed in three separate laboratories). The peptides C,E-dibenzyl- and T,C,E-tribenzyl-TYICEVEDZKEE were synthesized using conventional FMOC chemistry, in either a batch or automated format, cleaved in trifluoroacetic acid, rinsed with ether, and their structures confirmed by fast atom bombardment mass spectrometry after purifica-tion to greater than 90% by reverse-phase chromatography. Measurement of inhibition of HIV-induced cell fusion by CD4 peptides were done in the VB indicator cell assay as previously described by Lifson et al. (J Exp Med 164:2102, 1986). Quantitative syncytial-forming microassay (SFA) was used to determine the antiviral infectivity capacity of the peptides. Infectious cell center assay (ICC) as described by Nara and Fischinger (Nature 331:469, 1988), was utilized. To assess potential infectious activity associated with cells inoculated with virus, with or without peptide treatment, cells were collected from duplicate microtiter wells after being scored for syncytia in the quantitative syncytial-forming microassay, were pooled, washed once in 1 ml RPMI, suspended in two ml RPMI, and serially diluted in the same medium. One hundred μl of suspended cells, corresponding to 20-50,000, 2-5,000 and 200-500 cells, were then added to wells containing 50,000 fresh CEM-SS indicator cells on poly-L-lysine. Forty-eight hours later, plates were scored for the presence of syncytia, exactly as for scoring of the SFA. Thus, quantitation of secondary syncytia formed in the ICC assay gives an index of the number of infected cells that were present at the end of the preceding syncytial-forming assay (SFA).

Major Findings:

An extensive survey of the major genera of the animal kingdom has revealed a heat-labile, antiviral serum-based factor(s) present primarily in the rodent and feline family and minimal to no activities in the herbivores, carnivores, and select members of nonhuman primates including the chimpanzee. This activity is dilution-dependent (1:2-1:32) and loses its activity upon heating at 56°C. Further studies have detailed its absolute requirement for Ca++ and its capacity to bind the HIV-1 viral envelope. Furthermore, it is capable of preventing infection in vitro when added after virus adsorption. Further studies are underway to elucidate its nature and mechanism of action with hopeful and eventual application as an HIV-1 therapeutic and/or preventive modality.

Benzylated derivatives of peptides corresponding to residues 81 through 92 of the CD4 molecule inhibit fusion between chronically HIV-infected and uninfected CD4-positive cells, and syncytia formation in freshly infected lymphoid cell cultures (Lifson et al., Science 241:712, 1988). To further characterize this activity, recently an in vitro quantitative microassay for acute HIV infection was divided into two kinetic phases corresponding to the two general stages of the viral life cycle: viral infection and transmission of virus and viral protein products through cell contact or release of free virions. To evaluate peptide inhibition at specific stages of the viral life cycle, cultures were treated with peptide either continuously or only during the infection or transmission phases of the assay. CD4(81-92) peptide derivatives were completely efficacious, at 20-167 μM, to inhibit syncytium formation when present only during the infection phase,

only during the transmission phase, or when present continuously during both phases of the assay. When peptides were present during the infection phase of the assay, inhibition of syncytium formation correlated with decreased p24 expression and lack of secondary infectious cell centers when cells exposed to virus were washed and replated onto fresh uninfected indicator cells.

These data are consistent with complete inhibition of viral infection by peptide, when peptide is present during initial exposure to virus. Unexpectedly, parallel inhibition of syncytium formation decreased p24 levels and inhibition of secondary infectious cell center formation was also seen even when peptides were added as late as 48 hours after inoculation, during the transmission period of the assay. Since viral binding and penetration are believed to be completed well before 48 hours in this assay system, CD4 (81-92) peptide derivatives appear to exert a virustatic effect on cultures already infected with HIV-1, decreasing p24 production, cytopathicity, and cellular infectivity. These results suggest CD4-based antireceptor peptides have antiviral efficacy in mitigating established infection in vitro, and that interactions between the CD4-binding site of the HIV envelope glycoprotein and other cellular or viral components may be important in viral assembly or budding in addition to their established role in viral binding, entry, and HIV-mediated cell fusion.

Publications:

Lifson JD, Hwang KM, Nara PL, Dunlop NM, Fraser B, Paget M, Eiden LE. Synthetic CD4 peptide derivates that inhibit HIV-1 infection and cytopathology. Science 1988;241:712-5.

Patents:

Eiden LE, Nara PL, Fraser B, Hwang KM. US Patent 203,285,258,576: Anti-retroviral Agent, October 14, 1988.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP07148-06 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Studies on T-Cell Malignancies, Lymphomas and AIDS

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. C. Gallo	Chief	LTCB NCI
Others:	S. Z. Salahuddin	Senior Investigator	LTCB NCI
	S. Nakamura	Visiting Scientist	LTCB NCI
	P. Lusso	Visiting Fellow	LTCB NCI
	S. Sakurada	Guest Researcher	LTCB NCI
	P. Biberfeld	Guest Researcher	LTCB NCI
	W. Blattner	Chief, Family Studies Section	EEB NCI
	D. Ablashi	Senior Investigator	LCMB NCI

COOPERATING UNITS *(if any)*

Harvard University, Boston, MA (Judah Folkman); American Red Cross (Tom Maciag); Karolinska Institute, Sweden (Peter Biberfeld)

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Hematopoietic Cellular Control Mechanisms

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
6.0	3.0	3.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cell biology studies have focused on the role of human T-lymphotropic retroviruses (HTLV) in human T-cell malignancies, acquired immunodeficiency syndrome (AIDS) and human herpesvirus type-6 (HHV-6), a DNA virus. HTLV-I is a transforming virus, whereas human immunodeficiency virus (HIV-1) is cytopathic and kills the cells it infects. HTLV-I, HTLV-II, HIV-1 and HHV-6 have specific tropism for OKT4+ T-helper cells. The involvement of these viruses in neuropathy is being examined. HIV-1 has been shown to be associated with cells of monocyte-macrophage lineage. HIV-1 isolates obtained from different patients show genetic variations in the envelope region. Long-term cell cultures have been obtained from lung tissues and pleural effusions of AIDS patients with Kaposi's sarcoma (KS). These cells are of vascular origin which excrete a variety of factors that can promote the growth of different cell types by autocrine and paracrine mechanisms. Isolation of HTLV-II from patients with both B- and T-cells, and a comparison of the genomes of the new isolates with prototype HTLV-II-MO, indicates the presence of some heterogeneity among these isolates. HHV-6 has been shown to infect both B- and T-cells as well as megakaryocytes, and it appears that HHV-6 may be involved in immunosuppressive events associated with HIV-1.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

R. C. Gallo	Chief	LTCB NCI
S. Z. Salahuddin	Senior Investigator	LTCB NCI
S. Nakamura	Visiting Scientist	LTCB NCI
P. Lusso	Visiting Fellow	LTCB NCI
S. Sakurada	Guest Researcher	LTCB NCI
P. Biberfeld	Guest Researcher	LTCB NCI
W. Blattner	Chief, Family Studies Section	EEB NCI
D. Ablashi	Senior Investigator	LCMB NCI

Objectives:

It is anticipated that a greater understanding of the processes involved in the molecular control of cellular growth, differentiation, and the pathogenesis of human neoplasias, acquired immunodeficiency syndrome (AIDS) and Kaposi's sarcoma (KS) will lead to the ultimate goal of developing improved approaches to the prevention and therapy of human neoplasia.

Pursuing studies leading to an understanding of the origin of tumor viruses, how they acquire their oncogenic potential, how they interact with cells, and how they are transmitted throughout nature.

Leukocyte differentiation in vitro. Attempts are made to study exogenous and endogenous factors which affect the maturation and proliferation of normal and leukemic human bone marrow cells. The mechanisms involved in the maturation process are under study.

Growth of leukemic myeloblasts in liquid suspension and endothelial cells under the stimulus of a factor(s) produced by cultured human embryonic cells or human T-lymphotropic virus (HTLV-II)-infected cells.

The distribution of HTLV in human T-cell leukemia patients (HTLV-I), drug addicts (HTLV-II) and human immunodeficiency virus (HIV-1) from patients with AIDS from various parts of the world is being actively pursued.

Methods Employed:

A variety of cellular biological, biochemical and virological techniques are employed in the processing and culture of lymphocytes, endothelial cells and brain cells from patients with leukemias, lymphomas and AIDS. Retrovirus expression in the cell cultures is measured by immunofluorescence using monoclonal antibodies, reverse transcriptase (RT) assays and by electron microscopy. Other techniques include the use of enzyme-linked immunosorbent assays (ELISA), radioimmunoassays, Western blot analysis, neutralizing antibody assays, syncytia inhibition assays and inhibition of virus replication.

A number of techniques are being explored for the production of an effective vaccine against AIDS. These include preparation of large quantities of gp160 and fragments of gp120 for potential use in the study of immune response in different animal species and the testing of these antibodies for their capacity to neutralize HIV-1 replication. Synthetic peptides corresponding to the HIV-1 p17 and gp120 sequences are also being evaluated for potential usefulness as a vaccine.

Major Findings:

Spindle-like cells were successfully cultured for long periods of time from the involved tissue specimen taken from the lung, oral cavity, skin, orbit of the eye, tonsil, and pleural effusion obtained from AIDS patients with disseminated KS. The primary characteristics of these cells have been reported. Further characterization is in progress and will be reported in the near future. Functional studies of these cells with respect to their role in matrix formation or destruction with the help of collagenases, chemotactic and chemoinvasion capabilities have just been completed. The apparent conclusion of this study seems to be that these cultured cells are abnormal smooth muscle cells of vascular origin. These cells have been cultured with the help of a novel growth factor obtained from a T4+ transformed cell line developed by the infection of human umbilical cord blood leukocytes by HTLV-II. Purification of this factor is in progress. The effort to develop reagents is also in progress. These efforts, at present, are limited to the development of monoclonal and monospecific polyclonal antibodies to the long-term cultured AIDS-KS cells. Several monoclonal antibodies have been developed that react only with the AIDS-KS cells and not with the normal human umbilical vein endothelial cells or several other cell types.

The AIDS-KS cells are being further examined for the extent and type(s) of soluble mediators being expressed by them. As was expected and published in the previously mentioned studies, several soluble biologically active mediators have been identified, viz., IL-1B, TNF, TGFB, aFGF and bFGF. In addition, a new factor has also been identified through a collaborative study. This factor is in the process of purification.

These long-term cultured AIDS-KS cells have also been the subject of molecular analyses. These studies were basically directed at the identification of messages for the well-defined factors and initially to determine the extent of expression of proteins. In our continuing effort to biologically evaluate the AIDS-KS cells and their in vitro and in vivo functions, we discovered that these cells not only respond preferentially to the T4+ cell-derived factor supplement, but this effect is significantly enhanced by corticosteroids. This is universal for all the AIDS-KS cells. The study for the in vitro part has recently been completed. Since the initial success with a few specimens of lung biopsies and pleural fluids, we have expanded this to include KS specimens from several other sites, such as orbit, buccal cavity, tonsils and skin.

The in vivo systems that were used in previous studies have also been further improved. The use of whole egg for chicken chorioallantoic membrane assay has been discontinued. This was cumbersome, needed special facilities, and was difficult to document. This has been replaced by the "cracked egg" technique

which gives us the optical clarity of the petri dish and a flat chicken. This procedure is one of the state-of-the-art techniques used to assess angiogenesis induced by the effector molecule. The other in vivo assay which we developed previously and continue to use is the NCr nude mouse assay. We are now developing a "hairless" guinea pig model for the same purpose. This is much less expensive and gives us the advantage of testing several points (such as titration) on one animal. This effort is still in the preliminary stages but appears promising. We are standardizing the system for the use of special sponges for sustained signal release. These signals, in turn, will induce angiogenesis and other effects resulting in a developing KS-like lesion.

In addition to the above mentioned studies, a number of promising compounds (with proven anti-angiogenesis capabilities) are being tested. These include both seemingly toxic compounds, such as tetrahydrocortisone as well as nontoxic compounds such as SPPG. This effort is aimed at possible clinical application of a concept put forward in our previously published work. The hairless guinea pig will be very helpful in this phase of our work.

HHV-6:

The isolation of the novel human herpesvirus (human B-lymphotropic virus [HBLV] or HHV-6) from patients with a variety of hematological disorders was first reported by us in 1986. While the concomitant infection by HIV-1 and HHV-6 clearly could be coincidental, other observations suggest a possible synergistic role for HHV-6 in AIDS. In vitro studies have shown that HHV-6 can infect a number of other cell types. For example, we reported the infection of fresh peripheral blood lymphocytes with surface antigen. The infection results in a dramatic cytopathic effect on these cells. Furthermore, a number of cell lines consisting of T- and B-lymphocytes, as well as cells of other origins, e.g., megakaryocytes, could also be infected by HHV-6. Many of these same cell lines are also susceptible to infection by HIV-1. The dual infection of a susceptible cell line by HIV-1 and HHV-6 results in an accelerated loss of viable cells. While these observations are consistent with a possible synergistic activity, the actual mechanisms involved are unknown. HHV-6 could contribute directly by stimulating HIV-1 expression or by interacting with HIV-1 long terminal repeat affecting virus expression.

We have continued to study HHV-6. In addition to the development of information in the areas of biology and molecular biology, which have been reported, the following significant developments have taken place: 1) determination of the role of HHV-6 in lymphoproliferative diseases and chronic fatigue syndrome; 2) study of the interaction of HHV-6 and HIV-1 and HIV-2 in lymphoid cells (T4+) to understand the role of this virus, if any, in AIDS; and 3) a collaborative study was initiated for developing a series of monoclonals to HHV-6 proteins.

The role of HHV-6 as a primary etiological agent or as a cofactor in human disease was investigated. Recent findings show that approximately 14% of the heterophile-negative infectious mononucleosis (IM) cases were positive for IgM antibody for HHV-6 virus capsid antigen (VCA) only. The particular role of HHV-6 in chronic fatigue syndrome (CFS) patients is still under investigation. However, 51% of the patients had elevated antibody to HHV-6 VCA as compared to age- and sex-matched healthy donors. Active HHV-6 infection was detected in 9/12 CFS patients' peripheral lymphocytes, as directed by HHV-6 monoclonal antibody.

Normal donor lymphocytes (1/11) contained HHV-6 VCA-positive cells. These data are supported by in situ hybridization using HHV-6 DNA as a probe. Dual infection with HHV-6 and HIV-1 or HIV-2 of CD4+ cells (fresh cells or cell lines) showed enhanced killing, a significant increase in HIV-1 RT activity, and transactivation of HIV LTRs suggesting that HHV-6 may contribute directly or indirectly to the depletion of CD4+ T-cells in AIDS. An antigen capture assay has recently been developed as a consequence of the purification of several viral proteins and monoclonal antibodies. We need to test a reasonable panel of sera/body fluids to determine the levels of viral antigen in specific disease groups and normal people.

Publications:

Arya SK, Gallo RC. Human immunodeficiency virus type 2 long terminal repeat: analysis of regulatory elements. Proc Natl Acad Sci USA 1988;85:9753-7.

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Patents:

Ablashi DV, Salahuddin SZ, Gallo RC. (Pending): Infection of Transmission of HBLV into Glioblastoma and Megakaryocytes.

Gallo RC, Salahuddin SZ, Saxinger WC, Ablashi DV. (Pending): Testing for the Human B-lymphotropic Virus (HBLV).

Josephs SF, Gallo RC, Wong-Staal F, Salahuddin SZ. (Pending): Molecular Cloning and Clones of Human B-lymphotropic Virus (HBLV).

Salahuddin SZ, Gallo RC. (Pending): Cloned HTLV-I Producing Cell Line.

Salahuddin SZ, Gallo RC. (Pending): Human B-lymphotropic Virus (HBLV) Isolation and Products.

Salahuddin SZ, Gallo RC. (Pending): Virus Isolation and Viral Products of HBLV.

Salahuddin SZ, Nakamura S, Gallo RC. (Pending): Growth Factors and Kaposi's Sarcoma Derived Cloned Cell Lines.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP07149-06 LTCB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Molecular Biological Studies on Human Pathogenic Viruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	F. Wong-Staal	Research Microbiologist	LTCB NCI
Others:	R. C. Gallo	Chief	LTCB NCI
	S. Josephs	Research Chemist	LTCB NCI
	M. R. Sadaie	Senior Staff Fellow	LTCB NCI
	J. Rappoport	Staff Fellow	LTCB NCI
	V. Reyes	Visiting Fellow	LTCB NCI
	G. Franchini	Visiting Scientist	LTCB NCI
	M. L. Bosch	Guest Researcher	LTCB NCI

COOPERATING UNITS *(if any)*

Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (R. Franza); Duke University, Durham, NC (W. Greene); Walter Reed Army Institute for Research, Washington, DC (R. Redfield)

LAB/BRANCH

Laboratory of Tumor Cell Biology

SECTION

Molecular Genetics of Hematopoietic Cells

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
10.0	5.0	5.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The major efforts of our group at present are directed at studies on the HIVs. The following areas are addressed: (a) analysis of structure and function of the HIV-1 genome, with emphasis on the novel accessory genes of this virus; (b) analysis of the env gene, in detail, to define epitopes for neutralization, T4 binding, and viral cytopathic effect (CPE). Of relevance is our group's first demonstration of conserved and non-conserved domains in env; (c) molecular approaches to vaccine development. This work is currently carried out in collaboration with several industrial groups; (d) comparative analysis of the new virus subgroup HIV-2 and the related simian virus, SIV_{mac} as well as other HIV-1 and HIV-2 related isolates from several monkey species; and e) generation of a primate animal model to study the pathogenesis of AIDS and to test the efficacy of various vaccine approaches.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on the Project:

F. Wong-Staal	Research Microbiologist	LTCB NCI
R. C. Gallo	Chief	LTCB NCI
S. Josephs	Research Chemist	LTCB NCI
B. Ensoli	Research Microbiologist	LTCB NCI
M. R. Sadaie	Senior Staff Fellow	LTCB NCI
J. F. Rappoport	Staff Fellow	LTCB NCI
V. Reyes	Visiting Fellow	LTCB NCI
G. Franchini	Visiting Scientist	LTCB NCI
M. L. Bosch	Guest Researcher	LTCB NCI
S. Colombini	Guest Researcher	LTCB NCI
C. Gurgo	Guest Researcher	LTCB NCI
L. Marcon	Guest Researcher	LTCB NCI
S. Picciafuoco	Guest Researcher	LTCB NCI
J. F. Zagury	Guest Researcher	LTCB NCI
A. Buchbinder	Clinical Associate	COP NCI

Objectives:

The major objectives of this project are: (1) analysis of structure and function of the HIV-1 genome with emphasis on the novel accessory genes of this virus; (2) analysis of the HIV-1 *env* gene, in detail, to define epitopes for neutralization, T4 binding and viral cytopathic effect (CPE); (3) molecular approaches to vaccine development; (4) comparative analysis of the new virus subgroup HIV-2 and the related simian viruses; (5) HIV-2 in Rhesus macaque as an animal model to study human AIDS; (6) molecular cloning and structural analysis of HHV-6; (7) interaction between HHV-6 and HIV-1; and (8) molecular mechanism of pathogenesis of Kaposi's sarcoma.

Methods Employed:

Standard virological, molecular biological and recombinant DNA techniques of gene cloning, nucleotide sequencing, transfection, nucleic acid isolation, restriction enzyme mapping, Southern and Northern blotting, molecular hybridization and in situ hybridization are utilized in these studies.

Major Findings:

Functional Studies on tat and rev Proteins

We have continued our studies on the structure-function relationship of HIV-1 *trans*-regulatory genes with an emphasis on virus life cycle. In an early study based on site-directed mutagenesis, we showed that the cysteine residues in the *tat* *trans*-activator protein are essential for virus expression. This region of *tat* is proposed to have a possible role in metal/nucleic acid binding or dimerization. We have recently shown that conserved missense mutations with predicted cysteine to histidine changes within the proposed tetrahedral structure of the nucleic acid binding motif eliminate *tat* activity and virus expression. In contrast, one cysteine-to-histidine mutation outside the putative tetrahedron

had little effect on virus expression. Our studies suggest that although all but one cysteine residue in this domain are important for tat function, the region is unlikely to assume a prototype zinc-finger structure. Furthermore, we suggest that cysteine 31 is not metal-linked in the proposed dimer structure of tat. We have reconstructed our mutant clones in a version that allows a constitutive expression of each mutant under the control of heterologous promoter, i.e., that of cytomegalovirus immediate early gene. These constructs are being tested to elucidate the biochemical properties of the mutant proteins.

In collaboration with Pavlakis et al., Bionetics Research, Inc., we have generated several human established cell lines; each carries a tat- or rev-defective mutant provirus. These cell lines are being utilized as experimental models for HIV latency in vitro. We have found that photosensitization of tat- but not rev-defective cells via ultraviolet (UV) light irradiation rescues transmissible viruses. These revertant viruses display phenotypic characteristics of parental wild-type virus. These studies raise the question of whether natural exposure to UV light may contribute to the pathogenesis of AIDS. We propose that UV radiation of skin might directly or indirectly activate latent virus resident in human epidermal Langerhans cells and/or subepidermal lymphocytes.

The rev trans-regulator protein, though essential for virus replication, appears to play a pivotal role in the balance of HIV-1 gene over- or under-expression. Last year we reported that a rev-defective mutant HIV-1 expresses significantly higher levels of nascent viral RNA than wild-type. We, therefore, postulated that rev, like nef, may play a negative role in virus transcription. Extension of these studies clearly shows that rev exerts both a positive and a negative effect on virus replication, depending on the relative amount of rev supplied in trans. Studies with a reporter gene (CAT) linked to an HIV-1 LTR suggest that the cis-acting sequence responsive to rev down-regulation is localized to a region in the LTR upstream of the enhancer.

Anti-sense Oligonucleotides Directed Against HIV-1 Regulatory Genes

In collaboration with Makoto Matsukura of the Division of Cancer Treatment, NCI and Eric Wickstrom of the University of South Florida, we have investigated the effects of anti-sense methylphosphonate oligonucleotides directed against the tat (TAT0, TAT1, TAT3, TAT4, and TAT9) and rev (FWS.1 and FWS.2) messages of HIV-1. Oligonucleotides directed against the TAR site (TAT1) and in the 3' portion non-coding (TAT3) region of tat were found to inhibit virus replication, syncytia formation, and transactivation, and possessed low toxicity for $CD4^+$ lymphocyte cell lines, at levels up to 100 nM. Anti-sense oligonucleotides directed against the 5' splice-donor site and tat exon 1 initiation site lacked inhibitory activity. Both anti-sense wild-type and deletion mutant oligonucleotides directed against rev were able to protect SupT1 cells from infection, syncytia formation, and cytotoxic effects after infection or transfection with homologous cloned HIV-1 virus. Further experiments are underway to complete message walking experiments with these oligonucleotides and to explore the use of anti-sense oligo's as tools for the examination of functional sites of HIV-1 regulatory genes.

Mutagenesis of the Envelope per Variable Region (V3) of HIV-1

In collaboration with Steve Petteway and Lucy Ivanoff of Smith, Kline and Beckmann Laboratories, and with Jim Rusche and Scott Putney of Repligen Corp., a series of mutants with alterations or deletions of the V3 (HIVAC loop) region, identified as the immunodominant type-specific neutralizing epitope of HIV-1, have been constructed. One of these mutants shows a notably different phenotype from wild-type clones, with slower replication, differential replication in different T-cell lines, and reduced rates of syncytia formation and cytopathogenicity in permissive cell lines. Clones differing at specific residues also show reduced susceptibility to type-specific antisera raised against recombinant peptides (PB1, Sub2) corresponding to the wild-type sequences, complementing and extending previous observations upon the immunogenicity and neutralizing activity of anti-peptide sera made by Putney, Rusche, Matthews, Bolognesi, and others, as well as our own studies on type-restricted neutralization of molecular clones derived from HTLV-III_B.

Mutants currently constructed include HXB2/10 (pHXB2gpt with the Sal1 - BamH1 envelope insert of BH10), HXB2/10ala320 (replacement of the highly conserved proline with alanine), HXB2/10thr290 (replacement of the 290 serine residue with threonine), and HXB2/10Δ135 (a mutant with all but 6 residues of the loop deleted). An additional clone, HXB2/10arg313, which changed the BH10 313 serine to arginine, was found to have a deletion and is being reconstructed. The HXB2/10Δ135 mutant was found to be biologically inactive. As mentioned above, the HXB2/10ala320 mutant was found to have markedly altered biological properties, as well as a resistant neutralization phenotype to type-specific sera. Early metabolic labeling experiments suggest that envelope protein processing may be altered in this clone.

Further experiments are planned to examine smaller deletion mutants, express deleted mutants in E. coli and characterize antisera raised against the mutant envelopes. In addition, replacement of other regions of the loop with sequences from HIV-2 or SIV may yield additional insights into the function of the HIVAC loop.

Transactivation of HIV-1 by HHV-6

We have recently shown that human herpesvirus-6 (HHV-6) can coinfect with HIV-1 human $CD4^+$ T-cells leading to accelerated cell death, and factors in HHV-6 infected cells stimulate HIV-1 LTR-directed gene expression. We have now examined the mechanism of HIV-1 activation by HHV-6 and localized the cis-acting sequences of HIV-1 LTR responsive to trans-activation. In addition, we have studied the HHV-6-induced trans-activation of other retroviral promoters and found that HHV-6 trans-activates HIV-2 and SIV_{mac}-LTR linked CAT gene, but not HTLV-I LTR. We demonstrated that increased HIV-1 LTR trans-activation is obtained in HHV-6-infected cells coinfected with HIV-1 or cotransfected with the HIV-1 tat gene. Parallel increases of HIV-1-specific transcripts are seen by in situ hybridization in HHV-6/HIV-1 doubly infected cells as compared to single HIV-1 infection. Similarly, infection by HHV-6 increases the steady state level of HIV-1 LTR mRNA that parallels CAT enzymatic activity, suggesting a transcriptional and/or post-transcriptional activation. Analysis of the sequences necessary for HIV-1 LTR activation by HHV-6 revealed that they are distinct from those required for the tat response and map to a region of the HIV-1 LTR from -103 to -48. Furthermore, the HIV-1 enhancer sequence (-105 to -80)

is sufficient to confer HHV-6 inducibility to a heterologous promoter, and nuclear proteins activated or induced by HHV-6 infection specifically bind to the κB motifs of the HIV-1 enhancer region. Since HHV-6 and HIV-1 share similar $CD4^+$ cell tropism, our data suggest that HHV-6 infection may play an important role in the progression of HIV-1 infection to AIDS by increasing HIV-1 gene expression, viral replication and consequently accelerating the cytopathic effect in coinfected $CD4^+$ T-cells both by HIV-1 and by HHV-6 itself.

Cloning and Sequencing of HHV-6

We have developed methods for the growth and purification of HHV-6 and the cognate genomic DNA. The virus can be grown in large quantities (15 liters) in the HSB-2 cell line and concentrated by continuous flow centrifugation using 10-60% sucrose gradients. To purify the viral DNA, the virus is gently lysed and the DNA banded on CsCl gradients. BamH1-restricted HHV-6 genomic DNA yielded 16 fragments of which 11 are now cloned. The sequencing of these fragments which comprise approximately 50% of the HHV-6 genome is nearly completed. The cloning of the additional five high molecular weight fragments is in progress. These are being isolated from preparative agarose gels. Some changes in the restriction pattern of the viral DNA has been noted with passage of the virus. We have regrown the virus from early passage stocks for DNA isolation since the banding patterns were comparable to the prototype virus isolate. Fragments of the viral genome generated with Sal1 and Sst1 are being isolated for cloning. The two enzymes generated 21 and 17 fragments which were easily resolved on agarose gels. These fragments will be cloned and used to create a restriction map of the HHV-6 genome.

We have reported sequence similarity of the HHV-6 genome with the Marek's disease virus (MDV). Most of the hybridization seen under stringent conditions was due to a repeated sequence $(GGGTTA)_n$ shared by the two genomes. The sequence was repeated 26 times in the MDV genome and 60 times in the HHV-6 genome. The biological significance of these sequences is yet to be determined. The location of the sequences in the MDV IR_S and IR_L junction region is analogous to the "A" sequence in the HSV-1 genome and may function in packaging of the virus or inversion of viral sequences. It is interesting that the repeated sequence is identical to the human telomeric sequence.

Detection of HHV-6 Sequences in Tumors

A survey of hematopoietic tumors by Southern blot analysis showed detectable HHV-6 sequences in three B-lymphoma samples. These were obtained from patients with Burkitt's lymphoma, Sjogren's syndrome and nodular histiocytic lymphoma. A number of Hodgkin's and non-Hodgkin's lymphoma samples and a variety of leukemic specimens of both T- and B-cell types were negative for HHV-6 sequences by Southern blot analysis. An etiological role of HHV-6 in the Burkitt's lymphoma and the Sjogren's lymphoma is unlikely since HHV-6 could not be detected in the majority of Burkitt's samples and some tumor samples from the Sjogren's patient tested negative for HHV-6. Furthermore, in situ hybridization techniques showed that one cell in 10,000 could be detected in the positive Burkitt's lymphoma.

HHV-6 has been determined to be the etiological agent for Roseola Infantum by Yamanishi et al. (Lancet I:1065, 1988). The case for HHV-6 as the etiological agent for some cases of infectious mononucleosis was made by Dr. G. Krueger (University of Cologne, West Germany) in collaboration with members of this laboratory.

The polymerase chain amplification technique was adapted for the detection of low levels HHV-6 DNA. Peripheral blood DNA of a population of normal blood donors was tested and 32 of 57 tested (56%) were found positive. Correlation to serological positivity was not absolute since some DNA samples from persons who were serologically positive for HHV-6 were negative for HHV-6 sequences. On the other hand, some samples found positive for HHV-6 sequences had been obtained from serologically negative persons. Overall, HHV-6 infection in the population is common and no etiological association with any particular type of cancer has been found.

We found that 52 of 63 (83%) DNA samples from AIDS patients were positive for HHV-6 sequences by PCR technology. Investigations are continuing to determine the role, if any, of HHV-6 in the onset of AIDS.

The usefulness of PCR technology for determining an etiological role of HHV-6 in disease is limited. The prevalence of HHV-6 sequences in the normal population creates difficulties in interpreting data gathered from diseased populations for correlative purposes. Advances have recently been made in using PCR technology to quantitatively estimate the amount of HHV-6 DNA in sample preparations. This remains to be explored. In situ hybridization techniques coupled with histological staining may also be useful in certain cases. This is under development.

Evolutionary Relationship Among Primate Immunodeficiency Retroviruses

In the past year we obtained the entire nucleotide sequence of SIV_{mac} as well as of two HIV-2 viral isolates. These studies allowed us to conclude that the variability among HIV-2s is comparable to the variability observed among African but not American HIV-1 isolates. Such findings indicate that HIV-1 and HIV-2 have been in their respective African population for equivalent lengths of time. One important question is whether these retroviruses were transmitted to humans from monkeys. We identified high homology between SIV_{mac} and HIV-2 indicating the possible existence of monkey species in west Africa infected with a virus even more closely related to HIV-2 than SIV_{mac}. Similarly, a simian ancestor of HIV-1 could be identified in monkeys from central Africa where the highest prevalence of HIV-1 seropositive humans has been identified. Our plan is to study the genetic features of SIVs obtained from different geographical areas in Africa. We are presently analyzing five isolates from African green monkeys from Kenya and three isolates from a contiguous region (in collaboration with Drs. Markham and Zagury).

Characterization of SIV Regulatory Genes

We studied SIV_{mac} regulatory genes and their function. We analyzed the tat, rev, and nef genes which have been cloned as cDNA from SIV_{mac}-infected cells in a mammalian expression vector. The SIV tat gene trans-activates its own LTR as well as HIV-1 LTR, although less efficiently. SIV rev instead is not able to

replace the HIV-1 rev when transfected with a HIV-1 rev mutant. The nef gene of HIV-1 has been shown to have a negative regulatory function on viral replication. This effect appears to be mediated by inhibition of transcription from the viral LTR. We examined the ability of the SIV nef gene to down-regulate transcription of a reporter gene linked to the viral LTR and observed that SIV nef protein down-regulates gene expression to a lower extent than HIV-1 nef gene regardless of which LTR we used (HIV-1 or SIV_{mac}). Other properties of nef protein, like GTP bindings and GTPase activity, are under investigation.

Development of a Monkey Animal Model to Study Infection, Pathogenesis and Prevention of HIV Infection

We molecularly cloned two HIV-2 isolates and tested their infectivity in vitro and in vivo. The $HIV\text{-}2_{NIH/z}$ clone is infectious in vitro in fresh and neoplastic human T-cells but does not infect Rhesus macaques in vivo. Conversely, the $HIV\text{-}2_{sbl/isy}$ cloned virus is highly infectious in vivo. We inoculated, in collaboration with Dr. Markham, two juvenile Rhesus macaques one year ago and six more animals six months ago with $HIV\text{-}2_{sbl/isy}$. The first two animals seroconverted approximately two months after the inoculation and showed a decrease in their T-cell count and an inversion of the CD4/CD8 ratio six months after inoculation. Of the other six animals, three seroconverted and we could reisolate virus from the same animals a few months after inoculation. Our goals are: 1) to study the viral genetic determinants for infectivity and pathogenicity. We already constructed mutant viruses in an attempt to increase the infectivity and perhaps the pathogenicity of $HIV\text{-}2_{sbl/isy}$; 2) to immunize the animals using the HIV-2 envelope expressed in various systems and challenge the animals with various doses of viruses; and 3) to try to prevent horizontal and vertical transmission using the viral receptor (CD4) as a molecular decoy or other kind of molecular approach to replace viral replication in vivo.

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Patents:

Fisher AG, Ratner L, Gallo RC, Wong-Staal F. (Pending): Non-cytopathic Clone of Human T-Cell Leukemia Virus Type III.

Franchini V, Wong-Staal F, Gallo RC. (Pending): Molecular and Biological Characterization of a Replication Competent Human Immunodeficiency Type-2 Proviral Clone.

CONTRACT IN SUPPORT OF PROJECTS ZO1CPO5534-03 LTCB, ZO1CPO7148-06 LTCB

BIONETICS RESEARCH, INC. (NO1-CP7-3711-00)

Title: Procurement of Fresh Cells from Monocytes, Macrophages, and T- and B-Cell Lines

Current Annual Level: $198,873

Man Years: 1.76

Objectives: This contract provides supportive services in the supply of small quantities of T- and B-cells grown in tissue culture, partially purified IL-2, and radiolabelled and nucleic acids.

CONTRACT IN SUPPORT OF PROJECTS ZO1CPO5535-03 LTCB, ZO1CPO5536-03 LTCB, ZO1CPO5537-03 LTCB, ZO1CPO7148-06 LTCB

BIONETICS RESEARCH, INC. (NO1-CP7-3722-00)

Title: Provision of Tissues and Cells and Conduct of Routine Tests in Support of Tumor Cell Biology Studies

Current Annual Level: $754,651

Man Years: 4.91

Objectives: The major objectives of this contract are: (1) the use of immunofluorescence and radioimmunoassays to screen human T-cells and T-cell lines for viral structural protein expression; (2) the use of ELISA assays to detect antibodies against human retroviruses in serum from leukemia patients, AIDS and ARC patients, and normal donors; (3) to test culture fluids from short-term and long-term cultured cells for the presence of viral DNA polymerase activity; and (4) to test sera from patients with T-cell leukemia for antibodies to human type C RNA tumor virus (HTLV-I, -II, and -III).

CONTRACT IN SUPPORT OF PROJECTS ZO1CPO5536-03 LTCB, ZO1CPO5537-03 LTCB, ZO1CPO5538-03 LTCB, ZO1CPO5560-02 LTCB, ZO1CPO7148-06 LTCB, ZO1CPO7149-06 LTCB

BIONETICS RESEARCH, INC. (NO1-CP7-3723-00)

Title: Provision of Hematopoietic Cell Cultures, Growth Factors, and Type C Virus Proteins

Current Annual Level: $636,179

Man Years: 4.06

Objectives: The major objectives of this contract are: (1) to purify and supply factors that promote growth and differentiation of myelogenous leukemic cells and T-cells; (2) to purify the envelope and internal structural proteins of human and nonhuman primate type C RNA tumor viruses; (3) to prepare monoclonal antibodies against the purified structural proteins; (4) to provide cultured T- and B-cells from human cord blood, peripheral blood and leukemic cells; and (5) to prepare and supply radiolabelled cDNA and RNA probes from type C retroviruses.

CONTRACT IN SUPPORT OF PROJECTS ZO1CP05534-03 LTCB, ZO1CP05535-03 LTCB, ZO1CP05536-03 LTCB, ZO1CP05539-03 LTCB, ZO1CP07148-06 LTCB

BIONETICS RESEARCH, INC. (NO1-CP7-3725-00)

Title: Preparation and Purification of Viral Components

Current Annual Level: $201,209

Man Years: 1.20

Objectives: The major objectives of this contract are to prepare and supply large quantities of concentrated and purified human type C RNA tumor viruses.

CONTRACT IN SUPPORT OF PROJECTS ZO1CP05534-03 LTCB, ZO1CP05535-03 LTCB, ZO1CP05536-03 LTCB, ZO1CP07148-06 LTCB

BIONETICS RESEARCH, INC. (NO1-CP8-7213-00)

Title: Provision of Animal Facilities and Performance of Routine Experiments and Tests

Current Annual Level: $531,788

Man Years: 2.38

Objectives: This contractor provides animal facilities to house small and large animals, including rats, rabbits, goats and monkeys. The animals are used for the preparation of antibodies as well as for inoculation of tumor cells and virus preparations for tumorigenicity testing.

CONTRACTS IN SUPPORT OF PROJECTS ZO1CP05537-03 LTCB, ZO1CP05538-03 LTCB, ZO1CP05560-02 LTCB, ZO1CP07148-06 LTCB, ZO1CP07149-06 LTCB

BIONETICS RESEARCH INC. (NO1-CP8-7214-00)

Title: Provision of Purified AIDS Virus, Proteins and Subhuman Primate Facilities to Test Immune Response of Viral Antigens

Current Annual Level: $1,085,421

Man Years: 6.20

Objectives: The major objective of this contract is to supply purified HIV glycoproteins gp160 and gp120 for AIDS vaccine studies.

CONTRACT IN SUPPORT OF PROJECTS ZO1CPO5535-03 LTCB, ZO1CPO5537-03 LTCB, ZO1CPO5539-03 LTCB, ZO1CPO7149-06 LTCB

BIOTECH RESEARCH LABORATORIES, INC. (NO1-CP6-7731-00)

Title: Support Services in Virology, Tissue Culture, and Immunology

Current Annual Level: $337,263

Man Years: 3.37

Objectives: This contract is to provide supportive services in virology, immunology, and tissue culture. At the present time, these functions include: (1) detailed karyotypic analysis, including Giemsa banding; (2) evaluation of tumorigenicity of various cultured cells by inoculation into nude mice; (3) preparing small quantities of selected cells and retroviruses; (4) testing various tissue cultured cell specimens for mycoplasma contamination; and (5) analyses of sera for HTLV-I- or -III-related antibodies in sera of patients and normal donors by ELISA and Western blotting techniques.

CONTRACT IN SUPPORT OF PROJECTS ZO1CP05535-03 LTCB, ZO1CP07148-06 LTCB

BIOTECH RESEARCH LABORATORIES, INC. (NO1-CP7-3724-00)

Title: Preparation and Supply of Fresh and Cultured Mammalian Cells

Current Annual Level: $145,784

Man Years: 1.57

Objectives: This contract supplies well-characterized normal and neoplastic mammalian tissue culture cells and receives, processes, and distributes fresh human leukemic cells and tissues. Complete records are maintained on all biological materials handled under this contract.

CONTRACT IN SUPPORT OF PROJECTS ZO1CPO5538-03 LTCB, ZO1CPO5539-03 LTCB, ZO1CPO5560-02 LTCB, ZO1CPO7149-06 LTCB

REPLIGEN CORPORATION (NO1-CP7-1129-00)

Title: Preparation of Antisera Retrovirus Pool Antigens and Other Viral Components of HTLV-III/LAV

Current Annual Level: $0

Man Years: 4.07

Objectives: The major objective of this contract is to clone different regions of the *env* gene such as *tat*, *rev*, *nef*, in *E. coli* and baculovirus vector and to produce large quantities of the purified proteins after expression of these genes.

This contract terminated on December 15, 1988.

CONTRACT IN SUPPORT OF PROJECT ZO1CP07149-06 LTCB

REPLIGEN CORPORATION (NO1-CP9-5645-00)

Title: Supply Purified Recombinant Human Viral Proteins Produced in Insect Cells

Current Annual Level: $458,765

Man Years: 3.02

Objectives: The major objective of this contract is to produce and supply retroviral envelope proteins produced in insect cells.

CONTRACT IN SUPPORT OF PROJECT ZO1CP07149-06 LTCB

PAN DATA INC. (NO1-CP9-5626-00)

Title: Supply Purified Recombinant Human Viral Proteins Produced in E. Coli

Current Annual Level: $316,323

Man Years: 2.7

Objectives: The major objective of this contract is to produce and supply retroviral proteins produced in E. coli.

ANNUAL REPORT OF

THE LABORATORY OF TUMOR VIRUS BIOLOGY
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 through September 30, 1989

The Laboratory of Tumor Virus Biology (1) identifies and characterizes exogenous viruses associated with the initiation or progression of neoplasia in humans or in animals as models for human neoplasia; (2) elucidates the mechanisms by which viruses associated with naturally-occurring cancers may induce or initiate neoplasia; (3) characterizes and defines the biology and molecular biology of viruses associated with naturally-occurring carcinomas; (4) identifies and characterizes factors involved in viral and cellular gene regulation pertinent to carcinogenesis; and (5) elucidates and defines the cellular and molecular basis of the transformation in carcinogenic progression.

The Viral Oncology Section (1) studies the molecular biology and gene regulation of the papillomaviruses and mechanisms of papillomavirus-induced transformation and carcinogenic progression; (2) develops techniques for DNA-mediated gene transfer; and (3) carries out studies on extrachromosomal plasmid replication, recombination, and partitioning in mammalian cells.

The Cellular Regulation and Transformation Section (1) examines the role of DNA tumor viruses in carcinogenesis and neoplasia; (2) characterizes the cellular and biochemical alterations associated with the oncogenic transformation; (3) analyzes the specific interactions between cellular and viral proteins during oncogenesis; and (4) examines the effect of specialized cellular differentiated functions on viral gene expression.

Among the primary objectives of the Laboratory of Tumor Virus Biology is the evaluation of the potential role of the human papillomaviruses in carcinogenesis. To this end we have investigated the molecular biology of the papillomaviruses. A major focus of the Laboratory has been the molecular biology of the bovine papillomavirus type 1 (BPV-1), which is one of a subgroup of papillomaviruses which readily transforms a variety of rodent cells in tissue culture. This virus has served as the prototype for the systematic study of the molecular biology and genetics of the papillomaviruses. The BPV-1 genome encodes two independent transforming genes, one mapping to the E6 open reading frame (ORF) and the second to the E5 ORF. In transformed mouse cells, the viral genome remains as a stable multicopy plasmid, which is faithfully partitioned to the daughter cells at cell division. BPV-1 has characteristics making it an excellent model for the study of the cellular transformation and the viral functions important in a latent infection.

Bovine Papillomavirus

An important transcriptional regulatory circuit is encoded by the E2 open reading frames of all papillomaviruses. The circuit has been best studied in the bovine papillomavirus. E2 transactivation was first described by this

laboratory in 1985 and subsequent studies have shown that this open reading frame encodes at least three distinct proteins. In addition to the full-length E2 transactivator, the open reading frame encodes two transrepressors. One of these is expressed from an internal promoter and utilizes a methionine at nucleotide 3089; the second form can be translated from a spliced mRNA expressed from an upstream promoter and which consists of 11 amino acids from the E8 open reading frame fused to the carboxyl-terminus of E2. Genetic analyses have been carried out over the past year to genetically define the open reading frames that encode each of these three E2 proteins and to unambiguously assign the three proteins to their open reading frames. Functional studies have been carried out on the specific domains of the E2 open reading frame. The E2 open reading frame is relatively well-conserved among the papillomaviruses with two particularly well-conserved domains at the amino terminus and carboxy terminus of the full length protein. We have previously shown that the carboxy terminal region of 100 amino acids, which is shared by the E2 transactivator and the two repressor proteins, contains the specific DNA binding activity for these proteins. We have shown that the E2 proteins also form dimers and that the same conserved carboxyl terminal domain is sufficient for dimer formation. Dimer proteins are stable in the absence of DNA. The finding of the dimerization domain in a region common to each of the E2 proteins indicates that heterodimers can form and that this may reveal an additional mechanism of repression that could potentially result from the formation of inactive heterodimers consisting of transactivator and repressor species. Further genetic analysis of the E2 proteins has indicated that the amino terminal 200 amino acids, which are highly conserved among the E2 proteins, are necessary for the transactivator function. There is an internal region between the conserved amino terminal and conserved carboxy terminal domains which can be deleted with no effect on transcriptional transactivation.

The papillomavirus E2 transcriptional transactivator is representative of the class of transcriptional modulators in eukaryotes that activate transcription through direct binding to DNA sequences. In order to develop a system to genetically study the factors with which E2 may directly interact to mediate this transcriptional activation, we have introduced the E2 proteins into a lower eukaryote, saccharomyces cerevisiae. The BPV E2 transactivator can stimulate transcription from a yeast promoter containing E2 DNA binding sites ($ACCN_6GGT$). Whereas a single E2 binding site is sufficient for transactivation in yeast, a strong cooperative effect was observed with the two E2 binding sites. This contrasts slightly from studies in mammalian cells where two DNA binding sites are required for strong transcriptional transactivation. Deleted E2 proteins, lacking part of the transactivation domain or part of the DNA binding domain, failed to activate in yeast, similar to their behavior in mammalian cells. Replacement of the amino terminal region of the E2 transactivation domain with a synthetic amphipathic helix partially restored the transactivation function; however, it did not result in a molecule that exhibited cooperativity between neighboring E2 binding sites.

The BPV-E1 open reading frame encodes factors which are involved in DNA replication. Mutations which disrupt the translational integrity of the E1 open reading frame disabled the viral genome from replicating as a stable plasmid in transformed mouse cells and resulted in the integration of the viral genome into the host chromosome. Transcriptional studies have now been

carried out on a series of these BPV-1 mutants. Analysis of viral RNA within the transformed cells has revealed higher rates of transcription per viral genome in the cells harboring these mutated BPV-1 genomes. The levels of viral RNA coming from the viral piece of 89 promoter were found to be 15 to 35-fold higher in cells transformed by the E1 mutants compared to wild type BPV-1 transformants. These E1 mutants were found to be perturbed in their E2 transcriptional regulation, suggesting a possible explanation for the observed P-89 induction. Mutations throughout the E1 open reading frame, regardless of whether they were mapped to the replication domain or the DNA modulation function domain, were found to have this altered phenotype.

Studies have been continued on a line of transgenic mice harboring the complete BPV-1 genome. These mice heritably develop pathologies of the skin which are associated with the activation of the latent viral genome present and transmitted through the germ line. These mice develop abnormal skin characterized histologically by a benign proliferation of the dermal fibroblast. This fibromatosis is associated with an activation of transcription of the viral genome and the replication of the viral DNA as a stable plasmid within the cells. At approximately 8-9 months of age, some of these mice go on to develop fibroblastic tumors. The fibroblastic tumors of the dermis also contain extrachromosomal viral DNA which is transcriptionally active. No difference in patterns or levels of transcription or viral gene expression are noted within the tumor cells. These observations implicate cellular genetic changes in the later stages of tumorigenesis. Karotypic analyses have now been carried out on the fibromatoses and tumors. The normal cells of these mice are diploid. The fibromatoses are often aneuploid but demonstrate no specific chromosomal abnormalities. The fibrosarcomas, in contrast, show consistent abnormalities in one or both of two chromosomes (chromosome 8 and chromosome 14). These chromosomal abnormalities are not a direct consequence of the viral integration which has been mapped to chromosome 15 using in situ hybridization techniques. These results suggest that transgenic mice may be used to study the role of genetic changes in papillomavirus-associated tumorigenesis. Furthermore, such mice may permit the search for genes involved in tumor progression.

The BPV-1 genome encodes two viral oncogenes (E5 and E6). Studies in this laboratory have focused on the BPV-1 E5 protein. The E5 oncoprotein has been localized in the membranes of the Golgi apparatus and to a lesser extent, to the plasma membranes. The E5 protein is the smallest known to date and contains 44 amino acids. It is strikingly hydrophobic with a hydrophilic tail. The amino terminal two thirds of the E5 protein consist of hydrophobic residues which apparently facilitate association with the cellular membranes. Hydrophobicity is essential for the function of this domain and conservative amino acid substitutions within this region are still compatible with biologic activity. The 14 carboxyl terminal residues constitute a second domain of the E5 protein which contain several highly conserved amino acids. There are two cysteine residues which mediate E5 dimer formation. Substitution of the conserved cysteine residue abolishes E5 dimer formation as well as E5 transforming activity. Mutation of either cysteine alone abolishes transformation but not dimerization. This suggests that the E5 molecule may participate in important protein interactions other than homodimer formation that are crucial for biological function.

The complete lytic cycle of the papillomaviruses (including late gene expression) occurs only in the differentiated cells of a squamous epithelium. Malignant lesions and infected cells in culture do not produce virus. We have used bovine papillomavirus type 1 (BPV-1) as a model system for the study of papillomavirus late gene expression. A strong viral transcriptional promoter (called the late promoter) is used to transcribe the viral late genes and is active only in productively infected epithelium. Regulation of this promoter is not likely to be solely responsible for the control of late gene expression, however. Therefore, work in this laboratory has concentrated on identification of negative regulatory mechanisms which block late gene expression in BPV-1-transformed C127 cells. Our experiments suggest that transcription termination and mRNA turnover are also important mechanisms which regulate BPV-1 late gene expression. Specifically, nuclear run-off analysis of BPV-1 transcription in BPV-1 transformed C127 cells indicates that transcription of the BPV-1 genome is attenuated greater than 10-fold between the early and late polyadenylation sites, effectively favoring the use of the early polyadenylation site over the late polyadenylation site. In addition, transient expression assays with eukaryotic expression vectors have been used to map a putative transcription termination element to the 5′ portion of the late region. A second negative regulatory element has been localized to a 53 bp sequence in the late 3′ untranslated region and inhibits the expression of a heterologous gene when cloned into the 3′ untranslated region of that gene. This element most likely inhibits BPV-1 late gene expression in nonproductively infected cells by selectively destabilizing late mRNAs.

Human Papillomaviruses

A quantitative in vitro keratinocyte assay for the biological activities of the papillomaviruses has been developed using this assay. Most HPV DNAs tested have been found to stimulate proliferation of human keratinocytes. However, only with those HPV types associated with a high risk for malignant progression to cancer are capable of altering the keratinocyte response to challenges of terminal differentiation such as serum and calcium. These cells appear extended in their life span and may be immortalized. This assay has permitted a genetic dissection of the viral genes associated with transformation of human keratinocytes. Using HPV-16, we have been able to define two HPV genes (E6 and E7), which together are necessary and together sufficient for in vitro keratinocyte transformation. Studies with HPV-18 DNA indicate that the E6 and E7 genes of this virus are also sufficient for induction of this transformation.

We have extended our detailed genetic analysis of HPV-16 which is one of the human papillomaviruses strongly associated with specific anogenital carcinomas, specifically cervical carcinoma. We have previously shown that the E7 protein of HPV-16 has adeno E1a-like activities in that it can activate the adenovirus E2 promoter and can cooperate with an activated ras oncogene to transform primary baby rat kidney cells. Examination of the primary amino acid sequence of the 98 amino acid homologue HPV-16 and 17 protein reveals striking similarities of the amino terminus with two distinct conserved regions of adenovirus E1a. These regions in adenovirus E1a are involved in transformation and have been shown to correspond to the binding sites for the product of the retinoblastoma tumor suppressor gene (RB). The RB protein (p105-RB) is believed to be involved in cellular proliferation and complexing of this protein by the viral oncoproteins has important implications in terms

of the mechanisms by which these viruses transform cells. We have shown that the E7 protein of HPV-16 and of HPV-18 are capable of complexing with p105-RB. We are continuing mutational analysis of the E7 gene to define those amino acids required for stable complex formation with p105-RB. Additional studies are underway to examine other cellular proteins with which E7 may interact as well as cellular factors with which the E6 oncoprotein may interact.

Tyrosine Protein Kinases

During the past year the laboratory has continued to investigate the regulation and functions of the src family of tyrosine protein kinases in a variety of cellular systems. We have prepared numerous unique molecular and biochemical reagents for these studies and have analyzed in detail the expression of c-src, fyn, and lck in lymphoid cells. The results of these studies have defined, for the first time, the normal function of a member of this gene family and have identified a physiologically relevant substrate for a member of the src family.

Using murine and human T lymphocytes, we have discovered that the lck gene product is physically associated with both the CD4 and CD8 surface glycoproteins. These glycoproteins are important for T-cell signal transduction and development processes. Notably, the CD4 surface protein also acts as the cellular receptor for the human immunodeficiency virus. We have defined, biochemically and genetically, the cytoplasmic sequences of CD4 that are important for the interaction of the lck gene product and have been successful in reconstituting the lck-CD4 complex in murine fibroblasts. Additional experiments have demonstrated that the lck gene product is capable of directing CD4 signals to a component of the T-cell receptor by phosphorylating the zeta subunit on tyrosine residues. Phosphorylation of zeta on tyrosine residues is important for coupling T-cell signal transduction through the T-cell receptor with other biochemical pathways in the cells. These results indicate that the CD4-$p56^{lck}$ complexes are structurally distinct but functionally similar to the class of surface receptors which possess intrinsic tyrosine protein kinase activity.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP00543-11 LTVB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization of the Papillomaviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. M. Howley	Chief	LTVB	NCI
Others:	B. A. Spalholz	Senior Staff Fellow	LTVB	NCI
	P. Lambert	Biotech Fellow	LTVB	NCI
	A. McBride	Visiting Fellow	LTVB	NCI
	J. Byrne	Biologist	LTVB	NCI
	S. Vande Pol	Medical Staff Fellow	LP	NCI
	J. Schiller	Senior Staff Fellow	LCO, DCBD	NCI

COOPERATING UNITS *(if any)*

University of California, San Francisco, California (Doug Hanahan)
Pasteur Institute, Paris, France (Moshe Yaniv)

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Viral Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER:
5.0	4.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The papillomaviruses are a group of small DNA viruses associated with benign proliferative lesions in a variety of higher vertebrates that occasionally progress to malignancy. There are currently 59 distinct human papillomaviruses and six distinct bovine papillomaviruses. The productive expression of these viruses is linked to the differentiation program of these squamous epithelial cells and, to date, no culture system has yet been developed for the successful propagation of any papillomaviruses in the laboratory. The bovine papillomavirus type 1 (BPV-1) has served as the prototype of the papillomaviruses for unravelling its molecular biology. It is capable of inducing fibroblastic tumors in inoculated rodents and readily transforms a variety of rodent tissue culture cells in the laboratory. The unique feature of the BPV-1 transformation system is that the viral DNA can remain as a stable extrachromosomal plasmid within transformed cells. Our studies are designed to understand the molecular biology of the normal virus host-cell interaction with the hope of providing some insight into the viral and cellular factors that may be involved in carcinogenic progression. BPV-1 transgenic mice provide a model for the analysis of tumor progression with the papillomaviruses. Within the viral system, the E2 gene products have served as critical factors in regulating viral gene expression. The E2 open reading frame for BPV-1 encodes three distinct proteins with DNA binding properties. These proteins have both positive and negative effects on the regulation of viral gene expression. These proteins regulate viral gene expression by binding specifically to ACCN6GGT motifs which are conserved within the viral genome. The proteins can form dimers and models for the mechanism of the transrepression but the shorter E2 proteins involve competitive binding to the enhancer sites and subunit mixing by heterodimer formation. E2 transactivation has recently been demonstrated in yeast.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. M. Howley	Chief	LTVB	NCI
B. A. Spalholz	Senior Staff Fellow	LTVB	NCI
P. Lambert	Biotech Fellow	LTVB	NCI
A. McBride	Visiting Fellow	LTVB	NCI
J. Byrne	Biologist	LTVB	NCI
S. Vande Pol	Medical Staff Fellow	LP	NCI
J. Schiller	Senior Staff Fellow	LCO DCBD	NCI

Objectives:

1. To analyze the molecular biology of the papillomaviruses using the bovine papillomavirus as a model system.
2. To analyze the viral mRNAs expressed in BPV-1-transformed cells.
3. To localize the transcriptional elements in the viral genome involved in the control of viral gene expression.
4. To determine the factors, both viral and cellular, involved in the control of virus-specific gene expression for the papillomaviruses.
5. To analyze the "early" papillomavirus protein products expressed in transformed cells.
6. To determine the *cis* and *trans* functions required for autonomous extrachromosomal plasmid replication.
7. To determine the nature of the molecular events involved in the progression of a benign papillomavirus lesion into a malignant lesion.
8. To characterize the virally-encoded gene products involved in the transcriptional control of the papillomavirus genome, particularly the E2 transactivator and 3′ E2 repressor proteins.

Methods Employed:

1. Standard recombinant DNA technology for the cloning and propagation of papillomavirus hybrid plasmids.
2. Tissue culture.
3. Transcriptional analysis including Northern blotting, cDNA cloning into expression vectors, and nuclear run-off experiments.
4. DNA sequencing.
5. Immunoprecipitation, immunoblotting and immunofluorescence of viral proteins.
6. Transfer of DNA into mammalian cells using standard calcium precipitation, DEAE dextran or electrophoration technology.
7. Transgenic mice.
8. Genetic analysis of papillomavirus functions in *saccharomyces cerevisiae*.

Major Findings:

1. The E2 open reading frame (ORF) of BPV-1 encodes positive and negative acting factors that regulate viral gene expression. The full-length open reading frame encodes the transactivator and the two transcriptional repressors are repressed from the 3' half of the open reading frame. We have carried out a genetic analysis in collaboration with John Schiller, Laboratory of Cellular Oncology, to genetically define the open reading frames that encode each of the three E2 proteins. The full length open reading frame encodes a 48 kDa protein. The two smaller E2 proteins are 31 kDa and 28 kDa in size. Mutational analysis has revealed that the 31 kDa species results from translation initiation at an internal E2 ATG within the open reading frame. The smallest E2 protein species, the 28 kDa polypeptide is the product of the E8/E2 fusion gene and results from translation of a spliced mRNA. A manuscript describing these genetic analyses will be published in the Journal of Virology. We have previously shown that a carboxyl terminal region of 100 amino acids, which is shared by the E2 transactivator, and the two repressor proteins, contains the specific DNA binding activity for these proteins. We have shown that the E2 proteins also form dimers and that this same conserved 101 carboxyl terminal segment is sufficient for dimer formation. The dimer proteins are stable in the absence of DNA. The finding of the dimerization domain in a region common to each of the E2 proteins reveals that heterodimers can form and this may reveal an additional mechanism of repression that could potentially result from the formation of inactive heterodimers consisting of transactivator and repressor species of E2 proteins. Further analysis of the E2 transactivator shows that a conserved amino-terminal domain of approximately 200 amino acids is crucial for transcriptional transactivation. There is an internal region between the conserved amino terminal and conserved carboxyl terminal regions which can be deleted with no effect on transcriptional transactivation. A manuscript describing these studies was published in the Proceedings of the National Academy of Science.

2. The papillomavirus virus E2 transcriptional transactivator is representative of a class of transcriptional modulators that activate transcription through direct binding to cis-acting DNA sequences. We have introduced the E2 proteins into a lower eukaryote, saccharomyces cerevisiae. When expressed in yeast, the BPV E2 transactivator can stimulate transcription from a yeast promoter having E2 DNA-binding sites present in cis. Whereas a single E2 binding site was sufficient for transactivation, a strong cooperative effect was observed with two E2 binding sites. The level of transactivation was dependent on the position of the E2 DNA-binding sites in relation to the yeast promoter, with the maximal effect demonstrated when the binding sites were positioned upstream. This contrasts slightly from studies in mammalian cells where it appeared that the two DNA binding sites are required for transcriptional transactivation. Deleted E2 proteins, lacking part of the transactivation domain or DNA binding domains, failed to activate transcription in yeast, similar to their behavior in mammalian cells. Replacement of the amino terminal region of the E2 transactivation domain with a synthetic amphipathic helix partially restored the transactivation function; however, it did not result in a molecule that exhibited cooperativity between neighboring E2 DNA-binding sites. A manuscript describing these studies which

were carried out in collaboration with Dr. Moshe Yaniv at the Pasteur Institute was published this year in Genes and Development.

3. The BPV-1 E1 open reading frame encodes multiple functions involved in viral DNA replication. Mutations which disrupt the translational integrity of the E1 open reading frame disable the viral genome from replicating as a stable plasmid and result in the intergration of the viral genome into the host chromosome, often at a low copy number. Despite this low copy number of integrated viral genomes, BPV-1 E2 mutants transform rodent cells to anchorage independence very efficiently. Studies were carried out to provide insight into why this low copy number of replication defective mutants are capable of expressing an equal or greater transformation potential than wild type BPV-1. Analysis of viral RNA revealed higher rates of transcription per viral genome in cells harboring E1-mutated DNA than in cells containing wild type BPV-1 DNA. The levels of viral RNA from the viral P_{89} promoter were found to be 15- to 35-fold higher in cells transformed by E1-mutated DNAs compared with wild type BPV-1 transformants. This promoter controls expression of the viral E6 transforming gene and is normally regulated by the viral E2 gene products. These E1 mutants were found to be perturbed in their E2 transcriptional regulation, suggesting a possible explanation for the observed P_{89} induction. Mutation throughout the E1 ORF, in either the replication function domain or the DNA modulation function domain, were found to be altered in viral transcription.

4. We have continued our analysis of a line of transgenic mice harboring the complete BPV-1 genome. These studies are in collaboration with Dr. Doug Hanahan who is now at the University of California, San Francisco. These mice develop a variety of pathologies associated with the activation of a latent viral genome present and transmitted through the germ line. They develop large areas of abnormal skin with hair loss which is due to a generalized proliferation of dermal fibroblasts and atrophy of the overlying skin appendages. The DNA is extrachromosomal within the abnormal skin. The mice also develop dermal fibroblastic tumors in which the viral DNA is also extrachromosomal and transcriptionally active. The DNA is found integrated and not actively transcribed in the internal organs of these mice. Thus, the activation of BPV-1 transcriptional activity and of extrachromosomal viral plasmid replication correlate well with the dermal fibroblastic proliferation. These activities, however, are not sufficient for tumor formation. These observations strongly implicate cellular genetic changes in the later stages of tumorigenesis. A manuscript describing these studies has been published in Molecular and Cellular Biology. Chromosomal analysis of these lesions has indicated a number of specific chromosomal abnormalities which correlate with tumor formation. The normal cells in these mice are diploid. The fibromatoses are often aneuploid but demonstrate no specific chromosomal abnormalities. The fibrosarcomas, in contrast, show consistent abnormalities in one or both of two chromosomes, chromosome 8 (trisomy or duplication) and chromosome 14 (monosomy or translocation). The chromosomal abnormalities are not a direct consequence of the viral integration which we have mapped to chromosome 15 by in situ hybridization. These results suggest that transgenic mice can be used to study the role of genetic changes in papillomavirus-associated tumorigenesis and may direct the search for genes involved in tumor

progression. A manuscript describing these results is in press in the Proceedings of the National Academy of Sciences.

Publications:

Hermonat PL, Spalholz BA, Howley PM. The bovine papillomavirus P_{2443} promoter is E2 trans-responsive: evidence for E2 autoregulation. EMBO J 1988;7:2815-22.

Howley PM. General molecular biology of the papillomaviruses. In: DePalo G, Rilke F, zur Hausen H, eds. Herpes and papillomaviruses, Vol. II. New York: Raven Press, 1988; 41-52.

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McBride AA, Byrne JC, Howley PM. E2 polypeptides encoded by bovine papillomavirus type 1 form dimers through the common carboxy-terminal domain: Transactivation is mediated by the conserved amino-terminal domain. Proc. Natl Acad Sci USA 1989;86:510-14.

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Spalholz BA, Byrne JC, Howley PM: Evidence for cooperativity between E2 binding sites in the E2 transregulation of bovine papilllomavirus type 1. J Virol 1988;62:3143-50.

Spalholz BA, Howley PM. Papillomavirus-host cell interactions. In: Klein G, ed. Advances in viral oncology, Vol VIII. New York: Raven Press (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP00565-07 LTVB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transforming Activities and Proteins of the Papillomaviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	R. Schlegel	Chief, CRT Section	LTVB	NCI
Others:	V. Bubb	Guest Researcher	LTVB	NCI
	D. Goldstein	IRTA Fellow	LTVB	NCI
	J. Quintero	Microbiologist	LTVB	NCI
	M. Willingham	Chief, UCS	LMB	NCI
	J. Silver	Senior Investigator	LMB	NCI

COOPERATING UNITS *(if any)*

Department of Human Genetics, Yale University, School of Medicine, New Haven, CT (Dr. Daniel DiMaio)

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Cellular Regulation and Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Our laboratory's investigations are focused on the mechanisms by which the papillomaviruses transform both immortalized and primary cells in vitro. Specifically, we are studying the effects of human papillomavirus gene expression on the proliferation and differentiation of human keratinocytes as well as the individual and combined effects of the isolated viral transforming genes (E5, E6, and E7) on established and primary cells. We have shown that the E5 oncoprotein has two domains essential for biologic activity: a hydrophobic, membrane-anchoring domain which has no requirement for specific amino acid residues, and a 14 amino acid hydrophobic, carboxyl terminal domain containing several amino acids (including two cysteine-residues involved in E5 dimer and oligomer formation) which are essential for biological function. Using a baculovirus vector and a spontaneously derived cell line, we have also demonstrated that the E5 oncoprotein is present predominantly in the inner membrane leaflet of the Golgi apparatus and to a lesser degree in the outer leaflet of the plasma membrane. We have also developed a quantitative keratinocyte assay to analyze the effects of the HPV DNA and isolated HPV genes on keratinocyte proliferation and differentiation. These studies have shown that: (1) all types of HPV DNAs induce cellular proliferation; (2) only HPV DNAs associated with malignant progression induce altered cellular differentiation and cellular immortalization; and (3) altered cellular differentiation and immortalization are due to the combined effect of the E6 and E7 genes.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

R. Schlegel	Chief, CRT Section	LTVB	NCI
V. Bubb	Guest Researcher	LTVB	NCI
D. Goldstein	IRTA Fellow	LTVB	NCI
J. Quintero	Microbiologist	LTVB	NCI
M. Willingham	Chief, UCS	LMB	NCI
J. Silver	Senior Investigator	LMB	NCI

Objectives:

1. Generate polyclonal and monoclonal antisera against the transforming proteins of the human (HPV) and bovine (BPV) papillomaviruses.
2. Use specific antibodies to isolate and characterize the biochemical and biological properties of the papillomavirus oncoproteins, with particular emphasis on the BPV-1 and HPV-16 E5 proteins.
3. Perform mutagenesis of isolated HPV and BPV transforming proteins to determine functional domains.
4. Analyze the distinct states of keratinocyte differentiation following HPV DNA transfection (keratin biosynthesis, envelope formation, response to inducers of terminal differentiation, etc.).
5. Characterize the effect of individual HPV genes on the differentiated state of keratinocytes.

Methods employed:

1. Keratinocyte cell culture including selection assays and organotypic cell cultures.
2. Transfection of keratinocytes by lipofection and electroporation.
3. Animal tumorigenicity assays.
4. Recombinant DNA methodology for the construction of subgenomic BPV and HPV molecules.
5. Synthesis of mutant papillomavirus genes with oligonucleotides.
6. Expression of HPV and BPV transforming proteins in baculovirus expression vectors for protein purification and characterization.
7. Immunoprecipitation, immunoblotting, and immunofluorescence of viral proteins.
8. DNA and RNA hybridization.
9. FACS analysis of cell surface antigens.
10. Polyacrylamide and agarose gel electrophoresis.
11. DNA sequencing.
12. Site-specific mutagenesis using M13 vectors.
13. HPLC and FPLC of viral and cellular proteins.
14. Immunoaffinity chromatography.
15. Two dimensional gel electrophoresis.

Major Findings:

1. The E5 oncoprotein has a unique intracellular localization.

Using a baculovirus expression vector and an isolated mammalian cell line which overexpresses E5 protein, we demonstrated by immunofluorescence and immuno-electron microscopy that the E5 oncoprotein is predominantly localized in the membranes of the Golgi apparatus and that a lesser amount is present in the plasma membranes. The E5 protein has an unusual asymmetric orientation in cell membranes; the COOH-terminus faces intraluminally in the Golgi and extracellularly in the plasma membrane, suggesting that its conserved COOH-terminal domain may interact with regulatory membrane proteins such as growth factor receptors or other components of the signal transduction pathway.

2. The E5 oncoprotein consists of two distinct functional domains.

The amino-terminal two-thirds of the E5 protein consists of hydrophobic residues which apparently facilitate association with cellular membranes. Hydrophobicity, not amino acid sequence, is essential for the function of this domain and we have demonstrated that many conservative amino acid substitutions in this region are still compatible with biological activity. However, the insertion of strongly hydrophilic residues into the hydrophobic domain destabilizes the E5 protein and thereby abolishes its transforming activity. The 14 carboxyl terminal residues constitute the second domain of E5 and encode several highly conserved amino acids, including two cysteine residues which mediate E5 dimer formation. Substitution of the conserved cysteine residues abolishes E5 dimer formation as well as E5 transforming activity. Interestingly, mutation of either cysteine alone abolishes transformation but not dimerization. This suggests that either (1) the E5 molecule may participate in important protein interactions other than homodimer formations that are crucial for biological function or (2) the E5 dimers formed by only one cysteine residue do not confer appropriate conformation for interaction with the cellular substrate.

3. A newly developed keratinocyte assay identifies HPV types associated with cervical carcinoma as well as the HPV genes involved in in vitro transformation.

We have developed a quantitative in vitro keratinocyte assay for the biological activities of the papillomaviruses. The assay has demonstrated that many HPV DNAs (including those HPVs which are not associated with cervical carcinoma) can stimulate the proliferation of human keratinocytes. However, only those HPV types associated with progression to carcinoma are capable of altering keratinocyte response to inducers of terminal differentiation (e.g., serum and calcium) as well as immortalize these cells. This assay has also permitted us to demonstrate the modulatory effects of steroid hormones on keratinocyte transformation. More importantly, we have been able to define the two HPV genes which are necessary and sufficient for in vitro keratinocyte transformation: E6 and E7. This assay will be important in the future to define the biological effects of individually expressed E6 and E7 genes as well as potential cellular changes which accompany transformation.

Publications:

Burkhardt A, Willingham M, Gay C, Jeang K, Schlegel R. The E5 oncoprotein of bovine papillomavirus is oriented asymmetrically in Golgi and plasma membranes. Virology (In Press).

Horwitz B, Burkhardt A, Schlegel R, DiMaio D. 44 Amino acid E5 transforming protein of bovine papillomavirus requires a hydrophobic core and specific carboxyl-terminal amino acids. Mol Cell Biol 1988;8:4071-78.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP00898-06 LTVB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 charactars or less. Title must fit on one line between the borders.)*

Role of Human Papillomaviruses in Human Carcinogenesis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	P. M. Howley	Chief	LTVB	NCI
Others:	B. Werness	NRC Fellow	LTVB	NCI
	F. Thierry	Guest Researcher	LTVB	NCI
	J. Lichy	Biotechnology Fellow	LTVB	NCI
	K. Munger	Visiting Fellow	LTVB	NCI
	H. Romanczuk	IRTA Fellow	LTVB	NCI
	C. Yee	Biologist	LTVB	NCI
	J. Byrne	Biologist	LTVB	NCI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Viral Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
6.7	5.2	1.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

The papillomaviruses are associated with naturally-occurring cancers in a variety of animal species, including man. There are now 59 distinct human papillomaviruses (HPVs) which have been identified. Approximately 18 of these have now been associated with human genital tract lesions. Of these, HPV-6 and HPV-11 have been associated with a high percentage of benign genital lesions, and HPV-16, HPV-18, and HPV-33 have been found in a high percentage of cervical carcinomas. A number of human cervical carcinoma cell lines exist containing integrated HPV DNAs which are transcriptionally active. Integration of the viral genome in these cell lines and in tissues obtained directly from cervical carcinomas have demonstrated that often the HPV genomes are integrated in the malignant lesions. This integration event does not appear to demonstrate specificity with regard to the host chromosome but does indicate some specificity with regard to the viral genome. Integration often occurs in the E1 or E2 open reading frame, such that it disrupts expression of the E2 open reading frame. The E2 open reading frame of the papillomaviruses encodes a DNA binding protein which is involved in the transregulation of the viral promoters. Integration into the E2 open reading frame, therefore, results in the loss of this regulatory factor and the deregulation of the promoter upstream of the E6 and E7 open reading frames. The E6 and E7 open reading frames have been shown to be expressed on a regular basis in cervical carcinomas. The E7 open reading frame encodes a transcriptional transacting function which can activate the adenovirus E2 promoter. It can also cooperate with ras in the transformation of primary rat embryo cells. There are regions of the E7 gene of HPV-16 and of the E7 proteins of other genital-associated HPVs which are strikingly similar to domains of the adenovirus E1a gene product. The E7 protein of HPV-16 is able to bind the retinoblastoma gene product. This is also a characteristic of the transforming proteins encoded by other papovaviruses and the adenoviruses.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

P. M. Howley	Chief	LTVB	NCI
B. Werness	NRC Fellow	LTVB	NCI
F. Thierry	Guest Researcher	LTVB	NCI
J. Lichy	Biotechnology Fellow	LTVB	NCI
K. Münger	Visiting Fellow	LTVB	NCI
H. Romanczuk	IRTA Fellow	LTVB	NCI
C. Yee	Biologist	LTVB	NCI
J. Byrne	Biologist	LTVB	NCI

Objectives:

1. To analyze human squamous cell carcinomas from a variety of sites for the presence of HPV DNAs and the expression of HPV-specific mRNAs.
2. To characterize the HPV RNAs expressed in HPV-associated carcinomas.
3. To determine which viral genes are being expressed within the cervical carcinomas and cervical carcinoma cell lines.
4. To characterize the HPV-16 and HPV-18 genes which can transactivate transcriptional regulatory sequences within the viral genome and within the host cell.
5. To identify and characterize the HPV-16 and HPV-18 gene products involved in transformation and immortalization of a variety of cell types.
6. To determine the viral promoters active in benign HPV-16- and HPV-18-associated lesions and in cervical carcinomas.
7. To determine the cellular factors involved in regulating viral gene expression.
8. To identify the cellular factors which interact with the viral transcriptional, transregulatory, and transforming gene products.
9. To identify human genes that are inactivated in progression of benign papillomavirus lesions to malignancy.

Methods Employed:

1. Standard recombinant DNA technologies.
2. Northern blot analysis of RNAs.
3. cDNA cloning using expression vectors.
4. Immunoblotting and immunofluorescence of viral proteins.
5. DNA sequencing.
6. In situ hybridization.
7. Transient and stable DNA transfection techniques.
8. Cellular transformation using primary and established cell lines.
9. PCR analysis.

Major Findings:

1. In a continuation of our analysis of the HPV-16 gene products involved in transformation, we have identified, in a collaborative study with Ed Harlow

from Cold Spring Harbor Laboratories, a protein with which the E7 oncoprotein encoded by HPV-16 forms a stable complex. This protein is the product of the retinoblastoma tumor suppressor gene. Previous studies from other laboratories have shown that the P105-RB protein encoded by the retinoblastoma gene can form a stable complex with the transforming proteins of adenovirus (E1a) and SV40 (large T antigen). This protein is believed to be involved in cellular proliferation and complexing of this protein by the viral oncoproteins has important implications in terms of the mechanisms by which these viruses transform cells. A manuscript describing these data was published in Science. We are continuing a mutational analysis of the E7 gene of HPV-16 to define those amino acids required for stable complex formation with P105-RB. In addition, we are looking at the E7 proteins for a variety of other papillomaviruses for their ability to complex with P105-RB. At this point, we have evidence that the E7 proteins of all of the genital HPVs are capable of forming such a complex. This indicates that the E7 complexing with the P105-RB protein is, itself, not the factor that determines the malignant risk of the specific HPV type but rather is likely to be involved in the induction of benign cellular proliferation characteristics of all of these genital papillomaviruses. Additional studies are underway to identify other cellular proteins with which E7 associates.

2. Studies on the E2 transcriptional circuitry of HPV-16 have revealed that the P_{97} promoter of HPV-16, like that of HPV-18, is transrepressed by the E2 product of BPV. Similarly, the homologous E2 proteins of HPV-16 and HPV-18 can transrepress these promoters. Mutational analyses have been carried out on the E2 binding motifs located within the long control region (LCR) of HPV-16 and HPV-18 and have revealed that the major target for repression is the E2 binding site most proximal to the P_{97} promoters. Manuscripts are in preparation on these studies.

3. The HPV-16 has also been analyzed. Prior studies had indicated that this gene was an oncogene in that NIH-3T3 cells transformed by a plasmid which contained both the E6 and E7 open reading frames were more tumorigenic than the cell lines that were transformed by E7 alone. Further studies have now been carried out using primary human keratinocytes. In these studies, the E6 and E7 genes of HPV-16 together are necessary and sufficient for transformation of primary human keratinocytes. In the context of the full viral genome, mutations in either the E6 or the E7 open reading frames completely abrogated transformation of these cells. In contrast, mutations of E1, E2, E4 or E5 had no effect on transformation. The E6 and E7 genes were sufficient for transformation of the keratinocytes when they were expressed from a strong heterologous promoter. Studies have now been initiated to attempt to identify cellular factors with which the E6 protein interacts.

Publications:

Dyson, N. Howley, PM, Munger, K, Harlow, E. The human papillomavirus-16 E7 oncoprotein is able to bind the retinoblastoma gene product. Science 1989;243:934-7.

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Münger K, Phelps WC, Howley PM. Human papillomaviruses and neoplastic transformation. In: Bristol Myers Cancer Symposium. New York: Academic Press (In Press)

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Phelps WC, Yee CL, Münger K, Howley PM. Functional and sequence similarities between HPV16 E7 and adenovirus Ela. In: Current topics in microbiology and immunology. Stuttgart: Springer-Verlag (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05420-05 LTVB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Transformation by Polyomaviruses

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.B. Bolen	Microbiologist	LTVB	NCI
Others:	F. Gregory	Microbiologist	MB	NCI
	I.D. Horak	Medical Staff Fellow	MB	NCI

COOPERATING UNITS *(if any)*

Laboratory of Cellular Development and Oncology, National Institute of Dental Research, National Institutes of Health (K.C. Robbins).
Queens University, Kingston, Ontario, Canada (L. Raptis)

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Cell Regulation and Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The polyomaviruses comprise a class of small DNA tumor viruses within the papovavirus group of DNA viruses. Members of the polyomavirus class include polyomavirus of mice, simian virus 40 of monkeys, polyomavirus of hamsters, avian polyomavirus, and the JC, BK, and B-lymphotrophic viruses of humans. Of these viruses, the murine polyomavirus has been most thoroughly characterized with respect to the genetic elements and proteins involved in oncogenic transformation of mammalian cells. Oncogenic transformation of rodent cells by the murine polyomavirus requires the continued expression of the middle T antigen encoded by the virus. The middle T antigen is a membrane-associated phosphoprotein. All transformation-competent middle T antigens possess an associated tyrosine-specific protein kinase activity. One of the protein kinases associated with the middle T antigen is the product of the c-src gene. Two additional members of the src family of tyrosine protein kinases have also recently been shown to be associated with middle T antigen--the products of the c-yes and the fyn genes. These results indicate that the mechanisms through which polyomavirus transforms mammalian cells is by associating with and deregulating multiple cellular tyrosine protein kinases.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

J.B. Bolen	Microbiologist	LTVB	NCI
F. Gregory	Microbiologist	MB	NCI
I.D. Horak	Medical Staff Fellow	MB	NCI

Objective:

To determine the role of polyomavirus middle tumor antigen associated tyrosine protein kinases in polyomavirus transformation.

Methods Employed:

1. Standard recombinant DNA technology.
2. Cell culture
3. Cloning mammalian cells in culture.
4. Immunoprecipitation analysis and protein kinase assays.
5. Immunoblot analysis.
6. Phosphoamino acid analysis.
7. Generation of peptide-specific polyclonal antibodies in rabbits.
8. Transfer of DNA into mammalian cells.
9. Phosphoinositol kinase assays.

Major Findings:

Rabbit antisera raised against human FYN-specific peptides were used to evaluate the expression of the FYN gene product in normal and murine polyomavirus middle T antigen (MTAg)-transformed rat cells. These antisera were found to detect $p60^{fyn}$-MTAg complexes in lysates from the MTAg-transformed cells. The MTAg molecules associated with $p60^{fyn}$ were found to be phosphorylated on tyrosine residues at sites similar to that found in $pp60^{c\text{-}src}$-MTAg complexes.

Whereas the abundance of $p60^{fyn}$ was found to be less in the MTAg-transformed cells than in their normal counterparts, the specific activities of $p60^{fyn}$ molecules associated with MTAg and those derived from normal rat cells were found to be similar.

This is the final year of this project.

Publications:

Horak ID, Kawakami T, Gregory F, Robbins KC, Bolen JB. Association of $p60^{fyn}$ with middle tumor antigen in murine polyomavirus-transformed rat cells. J Virol 1989;63:2343-47.

Raptis L, Bolen JB. Polyoma virus transforms rat F111 and mouse NIH 3T3 cells by different mechanisms. J Virol 1989;63:753-58.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05481-04 LTVB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Biochemical Regulation of Tyrosine Protein Kinases

PRINCIPAL INVESTIGATOR *(List other professional personnal below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P. I.	J.B. Bolen	Microbiologist	LTVB	NCI
Others:	A. Veillette	Guest Researcher	LTVB	NCI
	I. D. Horak	Medical Staff Fellow	MB	NCI
	P. A. Thompson	Medical Staff Fellow	NCI-Navy	NCI
	J. Pyper	Guest Researcher	LTVB	NCI
	S. Simpson	Howard Hughes Fellow	LTVB	NCI
	E. M. Horak	Microbiologist	LTVB	NCI
	F. Gregory	Microbiologist	MB	NCI

COOPERATING UNITS *(if any)*

Medicine Branch, NCI (M. A. Bookman); NCI-Navy, NCI (F. Foss)

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Cellular Regulation and Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
6.0	5.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type Do not exceed the space provided.)*

Proto-oncogenes encode proteins that comprise a select group of cellular regulatory proteins whose mutation or aberrant expression can result in oncogenic transformation. More than half of all known proto-oncogenes encode tyrosine-specific protein kinases. With the exception of certain growth factor receptors, defining normal functions for proto-oncogene products has been elusive. The aim of the research within this project is to define the normal functions and regulation of the src family of tyrosine protein kinases--a family of tyrosine protein kinases whose members are all proto-oncogenes. Toward this goal, we have taken steps to prepare molecular and immunologic reagents to each of the seven members of the src family and to use these reagents to analyze the expression and activity of these genes and their protein products in normal and transformed cells and tissues. The results of our work over the past year have defined, for the first time, the normal function of a member of this gene family and have allowed us to determine, again for the first time, a physiologically relevant cellular substrate. Our results imply that other members of this family may possess similar functions.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

J. B. Bolen	Microbiologist	LTVB	NCI
A. Veillette	Guest Researcher	LTVB	NCI
I. D. Horak	Medical Staff Fellow	MB	NCI
P. A. Thompson	Medical Staff Fellow	NCI-Navy	NCI
J. Pyper	Guest Researcher	LTVB	NCI
S. Simpson	Howard Hughes Fellow	LTVB	NCI
E. M. Horak	Microbiologist	LTVB	NCI
F. Gregory	Microbiologist	MB	NCI

Objectives:

1. Analysis of the src family of tyrosine protein kinase members in normal and transformed cells.
2. Mechanism of regulation of the src family of tyrosine protein kinase members in normal and transformed cells.

Methods Employed:

1. Standard recombinant DNA technology.
2. Cell culture.
3. Transcriptional analysis of RNA.
4. Construction and analysis of cDNA libraries.
5. Polymerase chain reaction isolation of defined cDNAs.
6. Propagation of normal clonotypic T lymphocytes.
7. Fluorescence-activated cell surface analysis.
8. Differential isolation of normal peripheral lymphocytes and other cells by elutriation.
9. Generation of peptide-specific polyclonal rabbit antisera.
10. Immunoprecipitation and protein kinase assays.
11. Immunoblot analysis.
12. Phosphoamino acid analysis.
13. Two-dimensional peptide analysis.
14. One and two-dimensional gel electrophoresis.

Major Findings:

We have utilized three principal cellular systems in the study of the regulation and function of the src family of tyrosine protein kinases: human and murine T lymphocytes, human neuroblastoma, and human colon carcinoma.

Using T lymphocytes we discovered that one member of the src family, $p56^{lck}$, is physically associated with both the CD4 and CD8 T-cell surface glycoproteins and can be modified by serine kinases in response to T-cell activation signals. We have defined, biochemically and genetically, the cytoplasmic sequences of CD4 which are important for interaction with $p56^{lck}$ and have been successful in reconstituting the association between the lck gene product and CD4 in murine fibroblasts. Additional studies revealed that $p56^{lck}$ is capable

of directing CD4 signals to a component of the T-cell receptor that is involved in coupling T cell responses with other biochemical second messenger pathways. These results support the idea that the CD4-$p56^{lck}$ and CD8-$p56^{lck}$ complexes are structurally distinct but functionally similar to the class of surface receptors which possess intrinsic tyrosine protein kinase activity.

Using human neuroblastomas variant clones we found that the expression and activity of c-src is coordinately regulated with the differentiation state of the cells. This study demonstrated that the abundance of $pp60^{c\text{-}src}$ isoenzymes is proportional to the steady-state level of c-src mRNA. Using RNA isolated from normal human tissues we have cloned the human neuronal-specific cDNA of c-src. Analysis of representative clones revealed that c-src RNAs isolated from brain neurons contain an additional 18 base pair exons located between the third and fourth coding exons expressed in all other tissue types. Further analysis demonstrated that the two other members of the src family expressed in neurons; c-yes and fyn, did not possess similar alternatively spliced RNAs.

Using human colon carcinomas we found that the expression of the c-src and lck genes is regulated as a function of the differentiation state of the cells. In this study it was shown that differentiation of human colon carcinomas with sodium butyrate resulted in significantly decreasing the levels of both the c-src and lck mRNAs and their corresponding proteins. This decrease in expression of c-src and lck corresponded with the appearance of a more normal phenotype of the butyrate-treated cells.

Publications:

DeSeau V, Rosen N, Bolen JB. Analysis of phosphotyrosyl phosphatase activity in human colon carcinoma and normal colon mucosal cells. In: Lippman M, ed. Growth regulation in cancer. New York: Alan R. Liss 1988;36-54.

Foss FM, Veillette A, Sartor O, Rosen N, Bolen JB. Alterations in the expression of $pp60^{c\text{-}src}$ and $p56^{lck}$ associated with butyrate-induced differentiation of human colon carcinoma cells. Oncogene Res (In Press).

Herlyn M, Mancianti ML, Jambrosic J, Bolen JB, Koprowski H. Regulatory factors that determine growth and phenotype of normal human melanocytes. J Exp Cell Res. 1988;179:322-31.

Pyper J, Bolen JB. Neuron-specific splicing of c-src RNA in human brain. In: Perez-Polo JR, Maness PF, de Vellis J, Brugge JS, eds., Oncogenes in the nervous system. New York: Alan R. Liss (In Press).

Rosen N, Sartor O, Foss F, Veillette A, Bolen JB. Altered expression of src-related tyrosine kinases in human colon carcinoma. Cancer cells: the molecular diagnostics of human cancer. New York: Cold Spring Harbor Laboratory Press (In Press).

Thiele CJ, Cazenave L, Bolen JB, Israel MA. Developmentally regulated genes in neuroblastoma. Prog Clin and Biol Res 1988;271:185-94.

Veillette A, Bolen JB. Src-related protein tyrosine kinases. In: Benz C, Liu E eds. Oncogenes. Norwell:Kluwer Academic Publishers (In Press).

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Veillette A., Bookman MA, Horak EM, Bolen JB. The CD4 and CD8 T-cell surface antigens are associated with the internal membrane tyrosine protein kinase p56^{lck}. Cell 1988;55:301-8.

Veillette A, Bookman MA, Horak EM, Bolen JB. Signal transduction by the lymphocyte-specific tyrosine protein kinase p56^{lck} in T-cells. Adv Protein Phosphatases (In Press).

Veillette A, Bookman MA, Horak EM, Samelson LE, Bolen JB. Signal transduction through the T-lymphocyte CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56^{lck}. Nature 1989;338:257-9.

Veillette A, Horak ID, Bolen JB. Post-translational alterations of the tyrosine kinase p56^{lck} in response to activators of protein kinase C. Oncogene Res 1988;2:385-401.

Veillette A, Horak ID, Horak EM, Bookman MA, Bolen JB. Alterations of the lymphocyte-specific protein tyrosine kinase p56^{lck} during T-cell activation. Mol Cell Biol 1988;8:4353-61.

Veillette A, O'Shaughnessy J, Horak ID, Israel MA, Yee D, Rosen N, Fujita DJ, Kung H, Biedler JL, Bolen JB. Coordinate alteration of pp60$^{c\text{-}src}$ abundance and c-src RNA expression in human neuroblastoma variants. Oncogene (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05482-04 LTVB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Control of Papillomavirus Late Transcription

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	C. C. Baker	Senior Investigator	LTVB	NCI
Others:	L. M. Cowsert	Biotechnology Fellow	LTVB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Viral Oncology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The papillomaviruses cause benign and malignant lesions of squamous epithelia in higher vertebrates. The complete lytic cycle of these viruses (including late gene expression) occurs only in the differentiated cells of the squamous epithelium. Malignant lesions and infected cells in culture do not produce virus. An understanding of the regulation of papillomavirus gene expression and its relationship to the control of epithelial cell differentiation is necessary for the elucidation of the role of the papillomaviruses in carcinogenesis. We have used bovine papillomavirus type 1 (BPV-1) as a model system for the study of papillomavirus late transcription and its control. A strong viral transcriptional promoter (called the late promoter) is used to transcribe the viral late genes and is active only in productively infected epithelium. More recent experiments have indicated that transcription termination and mRNA turnover are also involved in the regulation of BPV-1 late gene expression. Specifically, nuclear run-off analysis of BPV-1 transcription in transformed C127 cells indicates that transcription of BPV-1 genome is attenuated greater than tenfold between the early and late polyadenylation sites, effectively favoring the use of the early polyadenylation site over the late polyadenylation site. In addition, a putative transcription termination element has been mapped to the 5′ portion of the late region. A second negative regulatory element has been mapped to the 3′ untranslated region and inhibits the expression of a heterologous gene when cloned into the 3′ untranslated region of that gene. This element most likely inhibits late gene expression in nonproductively infected cells by selectively destabilizing late mRNAs.

PROJECT DESCRIPTION

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

C. C. Baker	Senior Investigator	LTVB	NCI
L. M. Cowsert	Biotechnology Fellow	LTVB	NCI

Objectives:

1. To study the control of late transcription of papillomaviruses using bovine papillomavirus type 1 (BPV-1) as a model system.
2. To determine the viral and/or cellular factors involved in the trans-activation of the major late viral transcriptional promoter.
3. To identify the cis-acting sequence elements involved in the control of the major late promoter.
4. To determine if transcription termination between the early and late polyadenylation sites plays a role in the control of BPV-1 late gene expression.
5. To construct eukaryotic expression vectors suitable for the identification and mapping of cis-acting regulatory elements which lie within and 3' to transcription units.
6. To identify the cis-acting sequence elements in the late region of BPV-1 which may control late transcription through transcription termination, polyadenylation, and/or mRNA stability.
7. To identify the viral and/or cellular trans-acting factors which interact with late region sequence elements to control late transcription.
8. To set up an in vitro transcription system which terminates transcription within the BPV-1 late region and to use this system to assay cellular and/or viral transcription factors.
9. To develop an in vitro keratinocyte culture and differentiation system which is capable of supporting BPV-1 productive infection and to use this system to study regulation of late gene expression.

Methods Employed:

1. Tissue culture for the preparation of viral mRNA from non-productively infected cells.
2. Inoculation of calves by BPV-1 to generate fibropapillomas for the isolation of mRNA and factors produced during productive infection.
3. Standard isolation of DNA and RNA from cells and tissue.
4. Transcriptional analysis by cDNA cloning, Northern blotting, primer extension, nuclease S1 protection and nuclear run-off analysis.
5. Construction of eukaryotic expression vectors using standard recombinant DNA technology.
6. Control element mapping by the generation of successive deletions in viral control regions using the exonuclease III deletion system and subcloning of these deletions into eukaryotic expression vectors.
7. Functional analysis in vivo of transcriptional control elements by the transfer of recombinant plasmids into cells and the assay in vitro for recombinant gene products.

8. Selection of stable cell lines containing recombinant expression vectors for the analysis of transcription termination by nuclear run-off analysis and for the analysis of mRNA stability by actinomycin-D chase analysis.
9. Preparation of crude nuclear and cytoplasmic extracts from bovine fibropapillomas and BPV-1 transformed cells to assay for trans-acting factors in the in vitro transcription system and to identify DNA and RNA binding proteins.
10. Culture of bovine and human keratinocytes on rafts to allow full differentiation in vitro.
11. Analysis of the effects of keratinocyte differentiation on papillomavirus late gene expression in the in vitro raft system.

Major Findings:

1. RNA nascent chain analysis was used to determine if transcription termination within the late region of BPV-1 might contribute to the late transcriptional block in transformed C127 cells. These analyses revealed that the level of transcription of the 3' early region remains high in the 5' part of the late region and then is attenuated approximately fivefold about 1 kb downstream from the early polyadenylation site. Transcription continues to decrease throughout the rest of the late region and is down at least tenfold by the late polyadenylation site. This tenfold transcription attenuation in the late region would effectively favor the use of the early polyadenylation site over the late polyadenylation site and thus is one major component of the block to late transcription in the BPV-1 transformed cell and presumably also in the infected fibroblasts and basal epithelial cells of a fibropapilloma. There are still detectable levels of transcription near the late polyadenylation site, however, indicating that additional blocks must exist. These results are now in press in the Journal of Virology.

2. A new series of chloramphenicol acetyl transferase (CAT expression vectors [pOBCAT] has been designed for the mapping of negative regulatory elements. The pOBCAT vectors express levels of CAT in transfection assays which are approximately 100-fold higher than those obtained using pSV2CAT vectors. This facilitates transfection analysis in BPV-1-transformed C127 cells which typically give low levels of expression in transient assays and make these vectors ideal for the mapping of negative regulatory elements. An additional property of the pOBCAT vectors is an absolute requirement for a polyadenylation signal for efficient CAT expression, making them useful for assaying for the utilization of a polyadenylation signal. These vectors have proven useful for the mapping of transcription terminators and mRNA destabilization sequences (see below). A manuscript describing the cloning and properties of these vectors is currently in preparation.

3. The pOBCAT vectors are currently being used to map the cis elements responsible for transcription termination within the BPV-1 late region. An approximately 1 kbp late region fragment (n. 4452-5433), when cloned into the intron upstream of the CAT coding sequences in pOBCAT, dramatically inhibits expression of both CAT mRNA and CAT protein in transient expression assays in

BPV-1-transformed C127 cells. Since this fragment lies immediately upstream of the region of transcription termination described in (1), it is likely that this inhibition is due to transcription termination. Progressive 5′ and 3′ deletions into the BPV-1 fragment cloned into pOBCAT have mapped a putative transcription termination element to near nt 5000. Experiments are in progress to identify the cellular and/or viral factors which interact with this element and to attempt to determine how transcription termination is regulated during productive infection.

4. The pOBCAT vectors were used to demonstrate that the BPV-1 late polyadenylation signal can be efficiently utilized in transient expression assays in BPV-1-transformed C127 cells. Thus, it is unlikely that polyadenylation plays a significant role in the regulation of late transcription. However, a 53 bp sequence from the BPV-1 late 3′ untranslated region (3′ UTR) inhibits the expression of CAT mRNA and CAT protein when cloned in the sense orientation in the 3′ UTR of pOBCAT. This sequence element has minimal effect when cloned in the antisense orientation in the 3′UTR or when cloned in an intron upstream of the CAT gene in pOBCAT or downstream of the polyadenylation site in pOBCAT, suggesting that this cis element functions at the RNA level. The most likely mechanism of action of this element is destabilization of mRNA. Experiments are currently in progress to confirm this mechanism. Consistent with this hypothesis, however, is the observation that the sequence elements which are responsible for the rapid turnover of many unstable mRNAs (GM-CSF, c-myc, etc.) are present in the 3′ untranslated regions of these mRNAs and are AT rich. The 53 bp-negative element which has been identified in the BPV-1 late 3′UTR is approximately 75% AT. In addition, the late 3′ UTRs of the other papillomaviruses which have been examined are also very AT rich (70-80% AT). This suggests that similar negative elements may exist for all papillomaviruses and that RNA stability may play a role in the maintenance of viral latency.

5. The "raft" system for the in vitro growth and differentiation of keratinocytes is currently allowing us to get nearly normal differentiation of human keratinocytes in culture. Preliminary experiments are in progress to study the human papillomavirus infection of these cells with concentration on late viral functions. In addition to potentially providing an in vitro system for the propagation of papillomaviruses, this culture system may also serve as a system in which to assay papillomavirus cis regulatory elements as well as provide a source of trans-acting factors from differentiated keratinocytes.

Publications:

Baker C. Bovine papillomavirus type 1 gene expression. In Pfister H, ed. Papillomaviruses and Human Cancer. New York: CRC Press (In Press).

Baker C. Regulation of bovine papillomavirus type 1 late gene expression. In: Howley P, Broker T, Liss A. Papillomaviruses, UCLA symposia on molecular and cellular biology, New Series, Vol. 124, New York: Alan R. Liss (In Press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05518-03 LTVB

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Transformation and Gene Regulation of the Hamster Papovavirus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	J.B. Bolen	Microbiologist	LTVB	NCI
Others:	P.M. Howley	Chief	LTVB	NCI
	J. Pyper	Guest Researcher	LTVB	NCI
	R. Levis	IRTA Fellow	LTVB	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Tumor Virus Biology

SECTION

Cell Regulation and Transformation Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.0	2.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The hamster papovavirus (HaPV) was originally isolated from skin epitheliomas originating from hair follicle epithelial cells in Syrian hamsters. The HaPV virions are found in the keratinized layer of the epithelium from infected animals, but are not found in the basal layers. Thus, the maturation of this virus is limited to terminally differentiated keratinocytes, thereby resembling the tissue-specific tropism of the papillomaviruses. However, the morphology of HaPV virions, the DNA sequences of the HaPV genome, and the genetic organization of the HaPV genome clearly show that this virus is a member of the polyomavirus family. In contrast with other family members of the polyomaviruses and papillomaviruses, HaPV injection into newborn hamsters produces rapid and acute lymphomas and leukemias which are thought to be of T-cell origin. Thymectomy of the animals severely reduces the incidence of this disease but results in formation of sarcomas at the site of injection. Thus, the HaPV is capable of inducing tumors of lymphoid, mesenchymal, and epithelial origin in its natural host.

PROJECT DESCRIPTION

Names, Titles, Laboratory, and Institute Affiliations of Professional Personnel Engaged on this Project:

J. B. Bolen	Microbiologist	LTVB	NCI
P. M. Howley	Chief	LTVB	NCI
J. Pyper	Guest Researcher	LTVB	NCI
R. Levis	IRTA Fellow	LTVB	NCI

Objectives:

1. To identify and characterize the HaPV encoded proteins required for oncogenic transformation and tumor formation in hamster and other rodent cells.
2. To identify and characterize the HaPV cis-acting elements responsible for the control of early and late gene expression in different types of rodent cells and to determine what trans-acting viral encoded proteins are required for HaPV gene expression.

Methods Employed:

1. Standard recombinant DNA technology.
2. Cell culture, including primary keratinocyte preparation.
3. Transfer of DNA into rodent cells.
4. Transcriptional analysis of viral RNAs.
5. Polymerase chain reaction.
6. Generation of peptide and fusion-protein specific antisera in rabbits.
7. Immunoprecipitation and immune-complex protein kinases assays.

Major Findings:

During the second year of this project reagents and techniques have continued to be developed which will be needed for the analysis of HaPV early and late gene expression. These analyses and reagents will be important for exploring the relationship between specific viral gene expression and transformation.

In order to define which of the three predicted early region proteins is responsible for transformation, cDNA clones for each of these genes has been synthesized. These clones were generated using overlapping oligonucleotides to reconstruct the splice junctions predicted to be used for each of the early proteins. These cloned DNAs will be transfected into various cell lines including an immortalized hamster keratinocyte cell line and primary hamster keratinocytes. Reagents have been made for analyzing the early region proteins expressed in cells. Anti-peptide antibodies have been generated to the three early region proteins.

Two sensitive techniques to analyze the expression of rare mRNA species and to discriminate different messages synthesized from the same region of the genome have been prepared. Using RNase protection assays rare messages can be detected and quantitated and the polymerase chain reaction (PCR) greatly amplifies target sequences which can then be cloned and sequenced. Additionally, using this technique differentially spliced messages arising

from the same region of the genome can be detected. The PCR technique has been used to analyze the early region mRNAs present in two Fischer rat FR3T3 cell lines stably transfected by HaPV and apparently transformed by the viral DNA. These two cell lines were both found to be tumorigenic following injection into Fischer rats. PCR was used to determine which early messages are made in these two cell lines. The oligonucleotide primers used span the region encompassing all three predicted early region splice junctions. cDNA copies amplified using these primers would be expected to generate a 271 bp fragment from a large T (LT) message, a 621 bp message from a middle T (MT) message, and a 617 bp fragment from a small T (ST) message. The results of these experiments showed that only the 271 bp band representing the LT spliced message could be observed suggesting that no middle or small T mRNAs or proteins are made in these cells. These observations can be interpreted to indicate that one or more spontaneous cellular mutations were responsible for the observed cellular phenotype.

Initial studies on HaPV replication in infected hamsters indicate that virus proliferation is restricted to terminally differentiated hamster epithelial cells--the keratinocytes. To determine what factors regulate viral replication it is important to establish an in vitro culture system for HaPV. Previous attempts to develop an in vitro tissue culture system using a variety of primary and transformed hamster and rodent cell lines for studying virus replication have been unsuccessful. However, no work has been done on establishing an epithelial cell culture system. We have begun studies on HaPV replication by establishing a system for culturing primary hamster epithelial cells in vitro. We will use primary hamster fibroblasts from newborn hamsters as control cells.

To study the regulation of viral replication and potential maturation in keratinocytes we have generated, in addition to the reagents noted above, several molecular and biochemical reagents. These include: antibodies to the late viral gene products, nucleic acid probes which will be used to quantitate viral DNA replication and to determine the state of the viral genome in the infected cell, and nucleic acid probes to determine what RNA transcripts are being made from the late regions of the genome.

In summary, with the hamster keratinocyte cell lines now available and the cDNA clones specific for each of the early proteins, we now have a system in which immortalization and transformation functions, as well as the late gene expression control mechanisms, can be analyzed.

Publications:

None

ANNUAL REPORT OF

THE LABORATORY OF VIRAL CARCINOGENESIS
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 through September 30, 1989

FUNCTIONAL STATEMENT: The Laboratory of Viral Carcinogenesis (LVC) has as its charge the planning, development, implementation, and coordination of research programs on the etiology and mechanisms of carcinogenesis. In the past, particular emphasis was placed on delineating the roles, mechanisms and regulation of action of oncogenic viruses, virus-related and virus-associated genetic sequences, and gene products. Research efforts were conducted on virus-host relationships in virus-induced cancers, focusing on the detection and characterization of oncogenic viruses, their mechanisms of genetic integration and expression, and their modes of transmission in animals and man. The rapid technological development of eukaryote genetics and molecular biology has resulted in a synthesis of viral and cellular gene action during neoplastic processes. As a result, the research emphasis of the Laboratory has converged on the elucidation of regulatory events which are operative in human cancers and homologous animal models. In addition, the combined maturation of human genetics and molecular biology of infectious disease have permitted the implementation of experiments designed to study interaction of viruses and genetic structure of human populations. The multidisciplinary understanding of the neoplastic process, combining knowledge and technology from immunology, pathology, physiology, molecular biology, and genetics is the common character of the various research projects of the Laboratory, with a constant consideration of therapeutic opportunities. Activities of the Laboratory are conducted by in-house research and collaborative agreements with other research organizations.

SCIENTIFIC BACKGROUND AND SIGNIFICANCE: The combination of technological advances, and a number of coordinate, empirical observations, has dramatically altered the thinking of the scientific community on the molecular and genetic mechanisms of carcinogenesis. The procedures of molecular cloning, DNA sequencing, hybridoma production and somatic cell genetics have pushed the study of eukaryote genetic analysis from a speculative and interpretive discipline to the level of viewing gene action directly. A revolution in biological thinking and analysis is upon us, and these methodologies are being applied as rigorously to the dissection of carcinogenesis as to any other biological process. The contributions to the generalized journals (i.e., Science, Nature, and Cell) have experienced a quantum increase in definitive studies on the mechanism of carcinogenesis and transformation.

Several major advances are responsible for our changes in thinking, and these concepts and developments have influenced, and in part been influenced by, the research effort of the LVC, NCI. Among these generalized advances are: (1) The development of the concepts and properties of vertebrate "oncogenes." These loci are normal cellular genes which were initially described as transduced RNA segments in transforming retrovirus genomes and have also been discovered by focus induction after transfection of mouse 3T3 cells with

genomic DNA extracted from human tumors. The limited number of proto-oncogenes (circa 40) described to date has attracted considerable research emphasis over the past few years as an experimental opportunity to study neoplastic transformation directly from both genetic and molecular perspectives. (2) The normal functional role of several of these oncogenes during tissue development has recently been elucidated in several systems, including erb-B, fms, sis, jun, fos, ras, and a role in signal transduction, phosphorylation and in stimulation of cellular differentiation or proliferation. (3) Virtually all of the human oncogene homologs have now been chromosomally mapped to specific positions on the human gene map. These are in addition to the nearly 4,000 different loci comprising the human genetic map. In addition to the proto-oncogene loci, approximately 20 additional described loci are thought to participate in neoplastic transformation in man (e.g., growth factors, cell surface antigens, retroviral receptors, and integration sites, etc.). A dramatic advance has been the demonstration that certain human tumors with specific chromosomal rearrangements can be interpreted to involve the modulated regulation of cellular oncogenes by normally distant DNA regulatory elements which have been placed adjacent to the oncogenes by chromosomal rearrangement. (4) Despite a long history of unsuccessful attempts to isolate human type C retroviruses in a valiant effort by the former NCI Virus Cancer Program, two human diseases have been associated with exogenous human retroviruses. These are adult T-cell leukemia, which is etiologically associated with human T-cell leukemia virus-I and -II, and acquired immunodeficiency syndrome (AIDS), which has been serologically correlated with the development of antibodies to a retrovirus designated human immunodeficiency virus (HIV). (5) The study of animal models of certain of the homologous diseases in primates, rodents, and cats has provided terrific opportunities for reconstruction and analysis of the initiation and progress of mammalian tumors. For example, the AIDS models in primates (simian acquired immunodeficiency syndrome [SAIDS]) and in FeLV-infected cats (feline acquired immunodeficiency syndrome [FAIDS]) have modified significantly our interpretation of retroviral pathology to include broad levels of immune impairment in addition to (and possibly, in combination with) leukemogenesis.

The most significant findings of the LVC during this year are discussed below.

1. Expansion of the human gene map using physical mapping methods on genes which influence human tumorigenesis. The combination of two gene mapping technologies, somatic cell hybrid analysis and in situ hybridization, has been used in collaborative studies to genetically map 35 distinct human structural genes at the LVC. The rodent x human hybrid panels were developed using isozyme markers and high resolution G-trypsin banding from a group of over 1,000 hybrid clones. Southern analysis of hybrid DNA and in situ hybridization of tritiated probes to metaphase chromosomes have resulted in chromosomal localization of proto-oncogenes, growth factors, growth factor and retrovirus receptors, endogenous retroviral segments, and retroviral integration sites and members of the immunoglobulin gene superfamily. The collaborative gene mapping studies have served as the basis for several ongoing projects which relate to the genetic events involved in neoplastic transformation.

2. An association of HLA-DQ and HLA-DR1 with increased rate of progression to AIDS after infection with HIV. In studies of HLA antigens and disease association, HLA typing was performed on 260 individuals in the following groups: HIV-1 seronegative gay men, HIV-1 seropositive gay men, and patients with AIDS, Kaposi's sarcoma and opportunistic infections. In comparing differences in frequency of HLA antigens in these groups, prevalent disease risk was associated with HLA-DQW1. In a prospective study, HIV-1-infected individuals with the HLA-DR1 phenotype progressed to disease more rapidly than did individuals with other major histocompatibility complex class II antigens.

Mechanisms whereby HTLV-I infection might indirectly cause or contribute to the development of B-cell, chronic lymphocyte leukemia (CLL) are being investigated. B-cell lines established as spontaneous outgrowths from peripheral blood from HTLV-I-infected individuals with T-cell leukemia were found to produce growth factors that stimulate B-cell CLL cells and supported their growth in culture.

3. Demonstration of clinical pathology and immunodeficiency following inoculation of macaques with simian immunodeficiency virus (SIV/Mne). SIV/Mne viruses were isolated from macaque, SIV/Cat from mangabey, and SIV/Cae from African green monkeys. The three isolates were found to be different in properties of cell trophism and the antigen specificity of their viral proteins. SIV/Mne was administered to 3 species of macaques (31 animals) and produced disease with manifestation of immunodeficiency. An infectious molecular clone has been isolated from SIV/Mne and completely sequenced showing 94% homology with SIV/Mac, 87% with HIV-2, and 42% with HIV-1. Infection of macaques with this clone was associated with CD4+ cell depletion 60 weeks after inoculation. A type D retrovirus (SRV-2/WASH) was isolated from a macaque with simian AIDS. A recombinant vaccine virus that expresses the large envelope protein of SRV-2/WASH was constructed and used to immunize macaques. Sera from these animals neutralized SRV-2/WASH infectivity *in vitro*. Inoculation of animals with this construct appears to protect animals from disease development when challenged with infectious SRV-2/WASH.

4. Normal function of raf proto-oncogene defined. The structure-function analysis of c-*raf*-1 was completed, resulting in a model for the activity-regulation of the normally inactive enzyme. Physiological activation was characterized via a variety of extra- and intra-cellular mitogens leading to the following landmark findings: (a) c-*raf*-1 protein kinase is a substrate for several trans-membrane and intracellular tyrosine kinases as well as for protein kinase C (PKC); (b) tyrosine phosphorylation in a specific position activates the enzyme and PKC achieves a lesser degree of activation; and (c) in the case of the platelet-derived growth factor (PDGF) receptor, tyrosine phosphorylation occurs after direct binding of c-*raf*-1 protein to the PDGF-activated PDGF receptor kinase domain. This is the first example of a protein kinase cascade involved in mitogen signal transduction. Moreover, we demonstrated by cell fractionation and indirect immunofluorescence that activated *raf*, after initial translocation to the plasma membrane, moves to the perinuclear area, if not to the nucleus. Thus *raf* protein kinase acts like a shuttle enzyme that connects mitogenesis-related events at the plasma membrane to events in the nucleus. Consistent with these findings, we observed activation of transcription factor Ap-1 dependent gene transcription by activated, but not normal, C- and A-*raf* serine kinases. We are currently determining whether *raf* regulation of Ap-1 occurs by direct serine/threonine

phosphorylation. Modulation of transcription factor activity by raf family protein kinase was our original working hypothesis for the function of raf enzymes.

5. Role of raf in malignancy and raf-myc synergism. Overexpression and mutational changes of raf may contribute to the development of a variety of human neoplasms, including lung cancer, renal cancer, and synovial carcinoma. Two approaches yielded insight into these connections: (a) the promoter regions of C- and A-raf were characterized and revealed susceptibility to a variety of regulators, and (b) restriction fragment length polymorphism analysis demonstrated loss of heterozygosity of c-raf-1 in lung and renal cancer.

We have extended our previous observations of raf/myc synergism and observed that synergism is cell type dependent, that it occurs in growth factor abrogation as demonstrated with interleukin-3 dependent cells, leading to a general model for mitogen signal transduction from transmembrane tyrosine kinase receptors, and that it is essential for abrogation of c-myc translocations in plasmacytomas, indicating that activated c-myc is necessary, but not sufficient for, development of this tumor type.

6. Characterization of tumor promotion sensitivity genes in mouse and human cells. Evidence suggesting the involvement of promotion susceptibility genes in animal and human systems has come from the observation that animals can be bred for sensitivity to tumor promotion. Two genes, pro 1 and pro 2, that specify sensitivity to promotion of neoplastic transformation by tumor promoters in mouse epidermal JB6 cells have been cloned and sequenced and are being characterized with respect to mode of activation, regulation of expression, and nature of the gene products. Pro 1 is transcribed in vitro by polymerase III, yielding transcripts of 130 and 175 nucleotides. In vivo transcription yields an RNase protected pro 1 transcript of 130 nucleotides and a polymerase chain reaction detected transcript of 175 nucleotides. These transcripts contain B1-alu sequences and are contained within the biologically active sequence defined by deletion analysis. A new technique for site-directed mutagenesis has been used to generate pro 1 mutants defective for polymerase III transcription. These are being tested for P^+ activity. Such polymerase III transcripts can play a role in regulating mRNA splicing, transport, or translation. An aberrant polymerase III transcript, such as a P^+ active one, may cause altered post-transcriptional regulation of other genes.

DNA from the human nasopharyngeal carcinoma (NPC) cell line CNE_2 has been shown to be P^+ active in P^- JB6 cells and oncogenic in P^+ JB6 cells. Active and inactive pro 1 homologs isolated from the CNE_2 library are being sequenced to ascertain the mode of activation. Several sequence differences have been noted. A transforming gene whose activity is detectable in JB6 P^+ cells but not in NIH 3T3 cells has been cloned from NPC, and another is being cloned from a colon carcinoma cell line. In both cases transforming activity appears to be attributable to a non-ras gene. The NPC gene is 9 Kb in length and does not hybridize to any of 20 oncogenes or to human or mouse pro 1 or pro 2. The possibility that this apparently novel oncogene may cooperate with pro genes in the genesis of NPC will be investigated.

7. A role for PKC substrate p80 in promoter mediated transformation. The goal of the studies on "Signal Transduction in Tumor Promotion" is to determine the required biochemical events that occur between tumor promoter-receptor interaction and the activation of effectors of neoplastic transformation. Major signal transducing events being investigated include protein phosphorylation and transcriptional transactivation by AP-1 (jun/fos). A PKC substrate of 80 kDa has been found to be differentially phosphorylated in P^-, P^+, and neoplastically transformed JB6 cells, with little or no phosphorylated 80-kDa phosphoprotein (pp80) seen in transformed cells. Western analysis indicates that the progressive decrease of phosphorylated p80 is regulated at the level of synthesis with little or no p80 protein detectable in transformed JB6 cells. p80 has now been cloned from a P^- cell cDNA library. This p80 probe detects progressively decreasing p80 RNA during P^- to tumor cell progression. The possibility that p80 is a tumor suppressor will be investigated. Recent studies on 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-inducible genes have focused on those regulated by the trans-acting transcriptional factor AP-1 (jun oncogene). Transformation promoters TPA or epidermal growth factor induce AP-1 regulated gene expression in P^+ but not in P^- JB6 cells. This indicates that AP-1 regulated gene expression (1) may be required for tumor promoter induced transformation, and (2) in turn may be controlled by activated pro genes found in P^+ but not P^- cells. Evidence favoring the possibility of pro gene control of AP-1 function has come from the demonstration that introduction of activated mouse pro 1 into JB6 P^- cells cotransfers induced AP-1-dependent transactivation.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05326-07 LVC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

HLA Antigens: Structure, Function, and Disease Association

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
Others:	William Blattner	Chief, Family Studies Section	EEB	NCI
	James Goedert	Coordinator, AIDS Working Group	EEB	NCI
	Stephen J. O'Brien	Chief	LVC	NCI

COOPERATING UNITS *(if any)*

None

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Immunogenetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	0.6	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies are being carried out to determine the influence of major histocompatibility (MHC) genes and gene products on disease progression in HIV-1-infected individuals. HLA typing was performed on 400 individuals infected with HIV-1, some of whom had progressed to diseases (Kaposi's sarcoma and opportunistic infection) that are associated with infection. Approximately 300 of the individuals typed acquired the infection by sexual contact and the remainder is a result of therapeutic blood products. Some individuals have been followed over a period of years allowing analysis of HLA association with disease progression. In analysis at 60 months following infection, individuals with HLA-DR1 progressed to disease more rapidly than did individuals with other HLA phenotypes. Analysis at 78 months reversed the statistical significance of this phenotype as others with phenotypes other than HLA-DR1 progressed to disease. Sixty HIV-1 seropositive individuals in a hemophilic sib pair study were HLA typed and analyzed for disease progression as related to HLA haplotype. Preliminary analysis suggests that individuals who shared both haplotypes had common rates of disease progression. Sera from 30 hemophiliacs (10 developed AIDS, 10 HIV-seropositive AIDS-free and 10 HIV-seronegative) were obtained prior to seroconversion and subsequent yearly samples were tested for antibodies to HLA antigens. Individuals developing AIDS demonstrated increased reactivity to a range of HLA antigens. The frequency of reactivity to the panel tested occurred primarily with B-cells indicating development of antibodies to MHC class II determinants.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
William Blattner	Chief, Family Studies Section	EEB	NCI
James Goedert	Coordinator, AIDS Working Group	EEB	NCI
Stephen J. O'Brien	Chief	LVC	NCI

Objectives:

To determine function, structure and disease association of major histocompatibility complex (MHC) genes and/or their products. The studies are directed at the elucidation of genetic associations and potential genetic control of the immune response as it relates to disease process and etiology. Once markers are identified in the disease population, functional and biochemical studies are being performed in order to clearly define the genetic regulation of disease processes as it relates to immunologic response.

Methods Employed:

Standard HLA typing was performed using microcytotoxicity techniques. The technique for HLA-A,B,C has been described by Amos and Poole. The method for typing of the B lymphocytes for HLA-DR determinants was originally described by Mann et al. (Proc Natl Acad Sci USA 1975;72:5103-6). A total of 19 antigenic determinants controlled by genes at the HLA-A locus, 40 alloantigens at the B locus, 8 alloantigens at the C locus, 14 alloantigens at the DR locus, and 6 DQ antigens were tested for in the population study. The association of HLA types with disease was examined for significance by a number of statistical methods.

Major Findings:

This project continues to provide significant information relevant to expression of histocompatibility antigens, their genetic control and relationship to disease. HLA typing of the cohort of HIV-1-seropositive homosexuals, some of whom have developed Kaposi's sarcoma or opportunistic infection in the 5 years post infection, revealed an association of HLA-DR1 with the development of the disease. DQ1 was significantly increased in frequency in prevalent patients with AIDS compared to HIV-seropositive AIDS-negative individuals. In an analysis of incident AIDS in the HLA typed cohort of HIV-1-infected homosexuals at 78 months, there was no longer a statistically significant association of disease progression with HLA-DR1. However, the association of HLA-DQ1 with prevalent disease was strengthened. The majority of hemophiliacs (type A) in the United States were exposed to HIV by virtue of virus contamination of therapeutic blood products. Sera obtained from these individuals in late 1970, early 1980 (prior to exposure and seroconversion) and subsequent serum samples were screened for antibody to HLA antigens on a panel of T and B lymphocytes whose HLA phenotypes were representative of those found in the general population. These studies were

carried out in order to determine if loss of an immune response to a persistent and repeated challenge to HLA antigenic determinant provided by therapeutic blood occurred with the falling CD4+ lymphocytes that accompany disease progression. Contrary to expectations, the reactivity of the sera to the cell panel and thus to histocompatibility antigens increased, most predominantly in individuals developing AIDS. The reactivity patterns suggest the possibility that antibodies to MHC class II determinants participate in disease progression and/or results in autoimmune reaction. HIV-1-related disease progression was examined in 60 hemophiliac sib pairs that have been HLA phenotyped. In siblings where one individual developed AIDS, that individual's HLA haplotype was different from other infected sibs without the disease. In other siblings concordant manifestations of disease progression (not AIDS) was associated with shared HLA haplotypes.

Publications:

Mann DL, Murray C, Yarchoan R, Blattner WA, Goedert JJ. HLA antigen frequencies in HIV-seropositive disease free individuals and patients with AIDS. J AIDS 1988;1:13-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05328-07 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Immunologic Studies of the Human T-Cell Lymphoma Virus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
Others:	Mikulas Popovic	Medical Officer	LTCB	NCI
	William Blattner	Chief, Family Studies Section	EEB	NCI

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Immunogenetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.9	0.5	0.4

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The human T-cell leukemia virus, HTLV-I, is thought to be directly associated with adult T-cell leukemia (ATL) where virus can be directly demonstrated in the leukemic cells and indirectly with B-cell chronic lymphocytic leukemia (CLL). Studies are underway to understand the mechanism of malignant transformation of cells infected with this virus and to elucidate the mechanism that might indirectly influence carcinogenesis. B-cell lines infected with HTLV-I have been established from patients with ATL. The lines were cloned and HTLV-I-infected and non-infected clones established. The culture media from these clones were assayed for B-cell cytokines. CLL cells were obtained from patients who were HTLV seropositive and seronegative, and the culture media assayed for stimulating activity. The culture media from the HTLV-I-infected B-cell lines stimulated B-cell CLL cells and sustained growth of these cells for at least 28 days.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
Mikulas Popovic	Medical Officer	LTCB	NCI
William Blattner	Chief, Family Studies Section	EEB	NCI

Objectives:

These studies were designed to examine the biologic effects of infection with the human T-cell leukemia virus (HTLV-I). A number of studies from our laboratory and other laboratories have demonstrated that HTLV-I infection alters the dynamics of the immune response. HTLV-I has been demonstrated in tumor cells from patients with adult T-cell leukemia (ATL) and in nonmalignant T-cells in individuals with other neoplasms. In these individuals, the retrovirus was absent in the neoplastic cells. Experiments have been carried out to examine the possibility that HTLV-I infection was indirectly involved in the pathogenesis of malignancies other than the specific transforming event of the HTLV-I infection. We have demonstrated that B-cell chronic lymphocytic leukemia (CLL) cells from patients seropositive for HTLV-I are antigen committed, having the capacity to produce antibodies to HTLV-I proteins. We have investigated other mechanisms whereby HTLV-I infection of nonmalignant cells will indirectly contribute to carcinogenesis.

Methods Employed:

B-cell lines were established as spontaneous outgrowths of cells cultured from peripheral blood from patients with ATL. These cell lines were cloned to assess the uniformity of infection in all cells. Clones were found that were infected and noninfected. Culture media from these clones were compared for their ability to stimulate normal B-cells (measured by ^{3}H-thymidine incorporation) and compared with other known B-cell growth factors. Culture media from the HTLV-I-infected and noninfected B-cell clones were also tested for their ability to stimulate ^{3}H-thymidine incorporation in B-cell CLL cells from patients seropositive and seronegative for HTLV-I. Sustained growth of the CLL cells was also assessed using these culture fluids.

Major Findings:

Culture media from B-cell clones infected with HTLV-I produced a factor or factors which stimulate normal B-cells in the presence of anti-IgM antibodies. This stimulation was equivalent to or greater than other B-cell mitogens that were tested. These culture fluids stimulated ^{3}H-thymidine incorporation in B-cell CLL cells obtained from both HTLV-I seropositive and seronegative individuals. Viability and cell growth were also found in long-term culture of B-cell CLL cells indicating that a growth factor or factors for a malignant B-cell was being produced by the HTLV-I-infected cell.

Publications:

Clark JW, Gurgo C, Franchini G, Gibbs NW, Loffers W, Neuland C, Mann D, Saxinger C, Gallo RC, Blattner W. Molecular epidemiology of HTLV-I associated non-Hodgkin's lymphomas in Jamaica. Cancer 1988;61:1477-82.

Mann DL, LeSane F, Baumpas D, Blattner WA. HTLV-I infection and chronic lymphocytic leukemia. Nouv Rev Fr Hematol 1988;30:267-73.

Schneider M, Saal JG, Mann DL, Pawelec G, Schneider J, Schlote W, Wernet P. Respective T- and B-cell lymphomas in a married couple: in vivo activated T-cells lysing both tumor targets and concomitant humoral immune response pointing to a putative novel HLA class I restriction element related to HTLV-I. Int J Cancer 1988;41:548-56.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05367-05 LVC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Genetic Structure of Natural Populations of Past and Present

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI
	Rashid Aman	Visiting Associate	LVC	NCI

COOPERATING UNITS *(if any)* LCS, NIAAA, NIH, Bethesda, MD (D. Goldman); Natl. Zool. Park, Wash., DC (D. Wildt, M. Bush, L. Marker-Kraus); Program Resources Inc., Frederick, MD (W. Modi, D. Gilbert, D. Janczewski); Dept. Ecol. Behavior Biol., Minneapolis, MN (C. Packer); Univ. California Los Angeles, Los Angeles, CA (R. Wayne)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.4	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Population genetic analysis of human and animal populations has been used to study the genetic health and disease susceptibility of several species. The African cheetah has been shown to be a genetically depauperate relative to other species based upon a wide variety of methods: allozyme variants, two dimensional gel electrophoresis (2DE), allogeneic skin grafts, morphological asymmetry and DNA variants using mitochondrial DNA, major histocompatibility complex class I cDNA, and feline-specific hypervariable probes. Apparent physiological consequences of historic inbreeding depression are observed in reproductive norms and in a relatively high degree of infant mortality which has been decreased by subspecies hybridization. A molecular phylogeny of the great and lesser apes and man was derived based on genetic distance of 383 different proteins resolved by 2DE. A molecular phylogeny of the 37 species of the Felidae was constructed based on several molecular measures of evolutionary distance. Similarly, a consensus phylogeny of the Urisidae, Ailuropoda (giant panda) and Ailurus (red panda), was derived from distance matrices derived from three distinct molecular measures of genetic distance plus a cladistic analysis of isozyme and 2DE character data. A comparative analysis of cytological and linkage maps of mammals has indicated a noncontinuous tempo of chromosomal evolution in certain lineages (e.g., primates, felids) that are highly conserved in their chromosomal presentation, while others (rodents, lesser apes, canids) are chromosomally shuffled as if rapid saltatory cytological rearrangements occurred during the speciation events. A reconstruction of cytological rearrangements which have occurred during carnivore evolution has been achieved with particular emphasis on Canidae, Felidae, and Ursidae.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
Rashid Aman	Visiting Associate	LVC	NCI

Objectives:

(1) Use of molecular procedures to determine phylogenetic affinities and relationships between extant species of hominoid primates, felids, and selected carnivores. The derived topologies have important implications for heterologous embryo transfer and more generally for the ultimate resolution of the natural history of interacting gene systems that drive development and carcinogenesis. (2) Development of molecular procedures for assessing the genetic status of natural populations and for use in studying heritability of disease susceptibility, both congenital and etiologic. (3) The biologic resolution of adaptive strategies employed by rarely studied mammalian populations for defense against neoplastic and infectious etiologic agents that affect human populations.

Methods Employed:

The following techniques were employed: (1) cell culture procedures, (2) isozyme electrophoresis, (3) two-dimensional gel electrophoresis (2DE), (4) microcomplement fixation using heterologous rabbit antisera, (5) DNA hybridization, (6) high resolution cytogenetics procedures, (7) gene mapping procedures using somatic cell hybrids, and (8) statistical analysis of phylogenetic algorithms.

Major Findings:

1. Molecular evolution of Carnivora: Ursidae (bears) and Ailuropoda (giant panda). The taxonomic status of the giant panda and the lesser panda has been a biological puzzle since their description by western naturalists a century ago. We applied four independent molecular and one cytological method to resolve the phylogenetic position of the giant and red pandas, of the seven living Ursid species, and a few representative procyonids. The conclusions of these studies are summarized as follows. Between 30 and 50 million years before present (MYBP), the progenitors of the modern ursids and procyonids split into two lineages. Within 10 million years of that event (possibly at its inception), the procyonid group split into Old World procyonids and the New World procyonids. The red panda and giant panda clearly do not share a common ancestor after the ursid-procyonid split, emphasizing that the morphological similarities of the panda are probably the result of parallel retention of ancestral characters that may have been lost (for example, in the bear) after their divergence from the main line. At about 18 to 25 MYBP, the ancestor of the giant panda diverged from the ursid line. This event was at

least 20 million years after the initial divergence of the ursid and procyonid split. Near the time that the orangutan diverged from the African ape-human line (13 to 16 MYBP) the earliest true bear, Tremarctos (spectacled bear), split from the ursid line. The genus Ursus began its radiation into the ursine bears (brown, black and sun bears) 6 to 8 million years later (8 to 12 MYBP). A retrospective analysis of the karyology for this group indicated that the lineages leading to Tremarctos and Ailuropoda experienced a comprehensive fusion event of the primitive ursine acrocentric chromosomes.

2. The evolution of chromosome morphology in Carnivora is conservative, with the exceptions of global rearrangements in two families, Ursidae and Canidae. We have prepared high resolution G-banded karyotypes of over 90% of the species in three carnivore families, Ursidae, Canidae and Felidae, plus representative members of the other families. Within the Felidae, the 37 species all have either 18 or 19 pairs of chromosomes. Fifteen of these are invariant in all cats and 14 of these are found intact in all other carnivore families. This means we can derive an ancestral "carnivore" karyotype which we have done and it is very similar to the feline karyotype. We can also use the four variable feline chromosomes to generate a minimum distance cladistic evolutionary tree based on chromosome morphology. When this was attempted, the derived trees were consistent with the major clads predicted by the albumin immunological distance (AID) and allozyme genetic distance trees. When other carnivore families were examined the karyotypes were very homologous to the Felidae with the exception of Ursidae (discussed in Number 3) and the Canidae. The extensive chromosomal arrangements in the Canidae were not, however, beyond resolution. We have used the chromosomal morphology to reinterpret Canid evolution and a minimum distance evolutionary tree was derived by cladistic methods.

3. Molecular evolution in Carnivora: Canidae (dog family). The dog family, Canidae, consists of approximately 40 extant species which are generally classified in 10 to 12 genera. They have a worldwide distribution and their taxonomic relationship has been disputed. We have collected blood and tissue culture samples from 17 species representing each of the genera and used these materials for molecular evolutionary distance estimations. The products of 51 genetic loci for each species were analyzed by isozyme gel electrophoresis to generate matrices of genetic distance and character states. In addition, phylogenetic trees based upon parsimonious arranging of cytological exchanges were derived. Cladistic and phenetic evolutionary trees were produced with computer assistance. The results indicate that the Canidae can be divided into several monophyletic groups within which there are various degrees of phylogenetic similarity: the wolf-like canids, the South American canids, the Vulpes-like canids (foxes), Urocyon, Nyctereutes, and Otocyon.

4. Evidence for discordance of morphological and molecular evolution in free-ranging canids: Three sympatric jackals. Numerous studies have suggested that the extent of character divergence observed between two sympatric species reflects the intensity of competition for resources or space. However, the influence of time on divergence is often overlooked. Due to morphologic and developmental constraints, recently diverged species may remain relatively similar for some time, despite intense competition. Conversely, species with ancient divergence times may appear very distinct morphologically, although

competition is weak. Character divergence, as inferred from functional morphologic measurements, was examined in South American and African canids that are potential competitors. Differences in divergence time were determined by estimating the relatedness of these sympatric canids from data on mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLP's). Substantial character divergence was shown to occur rapidly, in less than 250,000 to 500,000 years, or to remain at low levels for extended periods of time.

Differences in the rate of intraspecific evolution were deduced by comparing mtDNA sequences, as inferred from RFLP's and direct sequencing between mtDNA genotypes of the East African black-backed jackal, Canis mesomelas elongae, and those of two other sympatric jackal species. The results were unusual for several reasons. First, mtDNA sequence divergence within several contiguous black-backed jackal populations is very large (8%). Previous intraspecific studies of terrestrial mammals have generally found values of less than 5% within a single population with larger divergence values most often occurring among mtDNA genotypes from geographically distant or isolated localities. Second, only 4 mtDNA genotypes were present in our sample of 64 jackals. The large sequence divergence observed among these mtDNA genotypes suggests there should be many more genotypes of intermediate sequence divergence. Finally, estimates of the rate of mtDNA sequence evolution differs by approximately threefold among black-backed jackal mtDNA genotypes, thus indicating a substantial heterogeneity in the rate of sequence evolution. The results preclude the presence of a constant molecular clock due to random fixation of selectively neutral or nearly neutral mtDNA sequence mutations in these species.

5. Demographic analysis of the captive cheetah population in North American zoological facilities reveals a population headed for extinction. The African cheetah has been bred in North American zoological facilities since 1956. The captive population has since grown to around 200 animals due to a combined increase in importation plus captive births. From 1982 to 1986, the captive birth rate declined by 50%, primarily due to a low frequency of breeding individuals in the population. The 1986 population had an effective breeding size of 28.1 in a total population of over 193 cheetahs. The incidence of infant mortality has been high (36.7%) relative to other zoo-bred species, perhaps as a consequence of the previously observed genetic impoverishment of the species. The combination of low fecundity, high infant mortality, and population dynamics indicates that the North American captive cheetah population is neither a self-sustaining nor a theoretically "viable population" as defined by Soule et al. (1986). Possible recommendations for improving captive cheetah propagation have been developed and communicated.

6. Molecular evolution in marsupials: Phenetic topology based on DNA hybridization. Unique sequence homology was compared between three major groups of Australian marsupials, Dasyridae, Macropodidae and Phalangeridae and the North American Didelphidae. DNA-DNA hybridization was performed using the S_1 nuclease digestion method. Matrices of melting temperatures (Tm's) and Tm's normalized for percentage of hybridization (TmR) were constructed for 11 different marsupial species. A phylogenetic tree was constructed from the two matrices using the UPGMA algorithm. The resulting topology was consistent

with previously derived trees from morphological and serological data. Divergence times were estimated from geological evidence which suggests that the divergence of the Australasian marsupials from their South American relatives occurred approximately 70 million years ago. The calibrated divergence times for the resulting topology suggest that the major marsupial Families and Genera had already formed in Australia by the lower Miocene (~25 million years ago). This is consistent with conclusions reached using AID. In addition, when correlated with the geological evidence, the data indicate a rate of DNA divergence which is proportional to similar values reported for several mammals and birds.

Publications:

Goldman D, Rathna Giri P, O'Brien SJ. Molecular genetic-distance estimates among the ursidae as indicated by one- and two-dimensional protein electrophoresis. Evolution 1989;43:282-95.

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O'Brien SJ, Nash WG, Martenson JS, Eichelberger MA, Wildt DE, Bush M, Goldman D, Wayne RK, Benveniste RE. Molecular biology and evolutionary theory: the giant panda's closest relatives. In: Warren L, Melselson M, eds. New perspectives on evolution. New York: Alan R Liss (In Press).

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05382-06 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 charecters or less. Title must fit on one line between the borders.)
Genes Involved in Preneoplastic Progression

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
Others:	John Seed	Senior Staff Fellow	LVC	NCI
	Cao Ya	Guest Researcher	LVC	NCI
	Paul Woolley	IPA Fellow	LVC	NCI
	Sylvie Poirier	Exchange Scientist	LVC	NCI
	Glenn A. Hegamyer	Health Science Officer	LVC	NCI
	Bruce Howard	Chief, Molecular Genetics Section	LMB	NCI

COOPERATING UNITS (if any) Hunan Med. College, Hunan, China (K.-T. Yao); PRI, Frederick, MD (R. Garrity); Dept. of Microbiology, Univ. of N. Carolina, Chapel Hill, NC (N. Raab-Traub); Dept. of Tumor Biology, M.D. Anderson Hospital, Houston, TX (D. Becker); BRI, Frederick, MD (W.K. Dowjat)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
4.8	4.1	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided.)

The aims of this research are to identify and characterize two classes of genes involved in multistage carcinogenesis. The first class are genes that specify susceptibility to tumor promoter-induced neoplastic transformation. The second class are genes that specify expression of tumor cell phenotype. Mouse promotion sensitivity (P+) gene pro 1, previously cloned from mouse epidermal JB6 cells and sequenced, shows evidence for encoding a polymerase III-catalyzed transcript, not a polymerase II transcript (mRNA). This pro 1 small RNA sequence identified by RNase protection as a 130-mer, falls within the region of P+ biological activity as defined by deletion analysis. Transcriptionally defective mutants of pro 1 have been produced by a novel site-directed mutagenesis procedure and these are being assayed for biological activity. Polymerase chain reaction analysis has identified the promotion-insensitive (P-) homolog of pro 1 and the sequence of this is being compared with that of the P+ active pro 1. Active and inactive human homologs of pro 1 isolated from a library of nasopharyngeal carcinoma (NPC) cells are also being compared at the sequence level, with the aim of identifying activating mutations. Several sources of NPC, both Epstein-Barr virus (EBV)-positive and EBV-negative NPC's, have been analyzed and found to show two DNA associated activities detectable in mouse JB6 recipients: (1) transfer of promotion sensitivity (P+ activity), and (2) transfer of oncogenic transformation (Tx activity). A novel transforming gene unrelated to the ras family or to some 20 other oncogenes has been cloned from NPC cells by human Alu screening of an NPC/JB6 transfectant DNA library. A similar Tx activity is also detected in colon carcinoma DNA and is being cloned using mouse JB6 recipient cells. With the cloning of the new NPC oncogene it now becomes possible to study the cooperation between genes involved in induction of cancer and genes involved in expression of tumor cell phenotype. The hypothesis that pro genes cause activation of oncogenes will be tested.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
John Seed	Senior Staff Fellow	LVC	NCI
Cao Ya	Guest Researcher	LVC	NCI
Paul Woolley	IPA Fellow	LVC	NCI
Sylvie Poirier	Exchange Scientist	LVC	NCI
Glenn A. Hegamyer	Health Science Officer	LVC	NCI
Bruce Howard	Chief, Molecular Genetics Section	LMB	NCI

Objectives:

To elucidate the nature of genetically determined events that are causally related to preneoplastic progression in mice and humans. To clone the genes involved in tumor promoter-induced progression to the tumor cell phenotype in JB6 mouse epidermal cells. To elucidate the structure and mode of activation of these genes. To learn how expression of these genes is regulated. To learn the functions of these genes and their products. To clone and characterize activated human homologs of promotion sensitivity genes. To clone and characterize a novel transforming gene(s) and its regulation by pro genes from both mouse and human tumors.

Methods Employed:

The following techniques are being utilized: (1) gene cloning techniques using sib selection and hybridization search routines; (2) calcium-phosphate DNA transfection followed by assay of sensitivity to promotion of anchorage-independence by the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA); (3) use of ligated dominant selectable markers; (4) generation of new recombinant DNA constructs; (5) restriction mapping; (6) sequencing the cloned genes by the Maxam and Gilbert or dideoxy technique; (7) computer-aided analysis of their structure and possible function; (8) Southern and Northern transfer techniques to analyze genome organization and expression of the pro and Tx genes; (9) RNase and S-1 protection to analyze size and sequence of RNA transcripts; (10) purification of nuclear and messenger RNA; (11) construction of genomic and cDNA libraries; (12) screening of libraries for pro gene homologs, for P^+-active sequences, and for transforming sequences using JB6 recipient cells; (13) calcium phosphate DNA transfection followed by assay of anchorage-independent, transforming activity (without TPA); (14) deletion analysis to define minimum biologically active sequences; (15) polymerase chain reaction amplification of DNA or reverse-transcribed RNA sequences; (16) generation of site-directed mutants by polymerase chain reaction; and (17) in vitro transcription assays.

Major Findings:

1. pro 1 appears to encode not a polymerase II transcript, but a small B1-Alu-containing polymerase III transcript. The use of single-stranded RNA probes for detection of pro 1 hybridizable RNA by Northern analysis revealed that a probe specific for detecting a predicted polymerase III-catalyzed transcript (but not one specific for a polymerase II transcript) hybridized to a small RNA band. Presence of a B1-Alu fragment on the pro 1 probe is essential for detection of this small RNA. A small RNA such as this could play a role in transcriptional or translational regulation of other genes.

2. The B1-Alu fragment of pro 1 and its 5′ and 3′ flanking sequences are essential for P^+ biological activity. pro 1 was subcloned into the pGEM vector, linearized, and digested with Exo III. Twenty mutants deleted at the 5′ end and 20 mutants deleted at the 3′ end were then tested for P^+ biological activity in a transfection assay. The deletion mutants capable of transferring the promotion-sensitive phenotype to P^- cells defined a 500-nucleotide sequence that preserved the integrity of the B1-Alu segment of pro 1.

3. RNase protection analysis of pro 1 hybridizing RNA detects a 130-nucleotide protected transcript. Hybridization of cytoplasmic RNA from P^-, P^+, and Tx JB6 cells with a full-length pro 1 RNA probe and hydrolysis with RNases A and H under stringent conditions that are expected to cut even single nucleotide mismatches, revealed a protected RNA for all three phenotypes of 130 nucleotides. This size RNase protected RNA could not have been obtained with known alternative candidate small RNA's such as 4.5S and 7S RNA's and is compatible with a faithful transcript of mouse pro 1. This 130-nucleotide pro 1 protected RNA is included within the boundaries of the biologically active sequence defined by deletion analysis.

4. Mouse pro 1 is transcriptionally active in an in vitro RNA polymerase III transcription assay. Two major transcripts are synthesized in the presence of 20 μg/ml but not 200 μg/ml α-amanitin. The molecular sizes are in the 130- to 180-nucleotide range and appear to be compatible with the size predicted for an RNA polymerase III transcript.

5. Production and analysis of RNA polymerase III promoter mutants of pro 1. The putative function of the B1-Alu fragment of pro 1 in the biological action of this gene is being assessed by site-directed mutagenesis. In order to assess the potential role of this transcript in the biological activity of pro 1, specific mutations have been introduced into the intragenic RNA polymerase III promoter element (B-box) of pro 1. These site-directed mutants were obtained by a novel and simple adaptation of polymerase chain reaction technology using mutated oligonucleotide primers and a 4.1-Kb plasmid containing a 1.2-Kb insert of pro 1 and a 2.9-Kb pGEM1 vector. The primers were located on complementary strands with each 5′ end adjacent to the other and polymerization proceeded in opposite directions around the plasmid. The product was a linear 4.1-Kb DNA fragment which, when ligated, regenerated the full-length circular plasmid with the primer-specific mutation incorporated at an approximately 90% efficiency. These plasmids are currently being tested for transcriptional activity (expected to be inactivated) in Hela cell extracts and for biological activity in P^+ transfection assays. Thus, the

hypothesis that pro 1 transcription is essential for its P^+ biological activity is being tested.

6. Polymerase chain reaction analysis of pro 1 transcriptional activity in vivo. In vitro transcription studies have demonstrated that pro 1 is transcribed by RNA polymerase III and gives two transcripts, one of approximately 130-nucleotides containing only the B1-Alu sequence and a "read-through" transcript that is 40-50 nucleotides longer. RNase protection studies of cytoplasmic RNA from P^-, P^+, and Tx JB6 cells (see #3 above) demonstrated a pro 1 protected 130-nucleotide transcript, a length expected for a B1-Alu transcript. A longer "read-through" transcript was not observed by RNase protection analysis. Evidence for the latter transcript has now been obtained in Tx JB6 cells using polymerase chain reaction technology to amplify low copy number RNA. Studies with the "read-through" transcript are being extended to other JB6 cell lines and will be correlated with biological activity.

7. Cloning of an inactive homolog of mouse pro 1. The inactive homolog of pro 1 was cloned from the promotion-insensitive cell line, Cl 30, by polymerase chain reaction. Nested primers were used to amplify a 460-bp fragment which contains most of the active region of pro 1 as defined by deletion analysis. The amplified fragment was subcloned into pUC19 and is currently being sequenced. Structural differences between the active and inactive homologs will be useful in identifying the mode of action of pro 1.

8. Comparison of P^+ active and inactive human nasopharyngeal carcinoma (NPC) sequences. One out of ten tested pro 1 homologous clones from the NPC cell line, CNE_2, turned out to be inactive. This particular clone, j, cannot be distinguished from active homologs i and b by restriction site polymorphism, heteroduplex formation, or Southern blot analysis using mouse pro 1, and various human molecular probes. This suggests that discrete changes, rather than gross rearrangements, may determine the P^+ activation of these pro 1 homologous sequences. The complete sequence analysis of activated and non-activated clones is expected to establish the mechanism of activation. Sequencing of the 1-Kb fragment of active clone, i, and the corresponding 1-Kb fragment of inactive clone, j, is presently underway.

9. Cloning of a transforming gene from the cell line, CNE_2. NPC DNA transfers an anchorage-independent transforming activity (without tumor promoter) to mouse JB6 P^+ cells. DNA from primary NPC/JB6 transfectants that is human Alu-positive has been used to generate secondary transfectants. A high proportion of these secondary transfectants show three characteristics: (1) human Alu positivity of DNA, (2) transforming activity of DNA on transfection, and (3) transformed phenotype of transfectants. A genomic library of tertiary transfectants has been screened to isolate the human Alu-positive sequence(s). DNA from secondary transfectants shows the absence of any characteristic human restriction fragments of Ki-ras, H-ras, or N-ras, as well as a lack of transforming activity in the NIH 3T3 focus assay. Human Alu screening of the genomic library of tertiary NPC/JB6 transfectants has led to the isolation of a novel 15-Kb, oncogenically active sequence that does not hybridize to any of some 20 known oncogenes or to pro 1 or pro 2.

10. Concurrent expression of Epstein Barr virus (EBV) is not necessary for expression of either P^+ or Tx activity of nasopharyngeal carcinoma DNA. A series of NPC samples, including EBV-negative cell lines and EBV-positive nude mouse carried NPC's, as well as tumor biopsies, were analyzed for DNA associated transfer of P^+ activity to P^- JB6 cells and for transfer of transforming (Tx) activity (tumor promoter independent) to JB6 P^+ cells. All NPC DNA's showed both P^+ and Tx activity, suggesting that NPC pro genes and oncogenes function independently of concurrently expressed EBV genes.

11. DNA from human colon adenocarcinoma cell lines transfers a non-ras transforming activity. DNA from the human colon adenocarcinoma, HT-29, and DLD cell lines transforms P^+ JB6 clone 41 cells. Some 25 clonal transfectant cell lines were established from the primary round of transfection. Of those containing human Alu sequences, a high proportion showed a transformed phenotype and DNA that transferred anchorage-independent transforming activity. Both secondary and tertiary DLD/JB6 transfectants were found to be tumorigenic in nude mice in contrast to the recipient JB6 cells. This transforming activity is probably not due to the ras family because (1) DNA of neither parental cell line transforms NIH 3T3 cells; and (2) Western blot analysis showed that the p21 protein in the parent cells showed no overexpression and no change in migration, i.e., no clear evidence of altered ras. This transforming activity is being cloned.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05383-06 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Membrane Signal Transduction in Tumor Promotion

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.)* *(Name, title, laboratory, and institute affiliation)*

PI:	Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
Others:	Stephanie Simek	IRTA Fellow	LVC	NCI
	Lori Bernstein	Biologist	LVC	NCI
	Elia Ben-Ari	IRTA Fellow	LVC	NCI
	Bonita M. Smith	Special Volunteer	LVC	NCI

COOPERATING UNITS *(if any)*
Swiss Inst. for Exp. Cancer Res., Lausanne, Switzerland (P. Cerutti), NINDS, NIH, Bethesda, MD (D. Kligman); NIMH, NIH, Bethesda, MD (J. Patel); U. South Calif., Los Angeles, CA (P. Vogt); Univ. Calif., San Diego, CA (M. Karin)

LAB/BRANCH
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SECTION
Cell Biology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
3.6	2.4	1.2

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The goal of these studies is to determine the required biochemical events that occur between tumor promoter-receptor interaction and the activation of effectors of neoplastic transformation. Candidate second messengers being addressed include protein phosphorylation by protein kinase C (PKC) and PKC-regulated trans-activation of gene expression. A C-kinase substrate of 80 kDa has been found to be differentially phosphorylated in promotion-resistant (P-), -sensitive (P+), and neoplastically transformed JB6 mouse epidermal cells, with little or no phosphorylated 80-kDa phosphoprotein (pp80) seen in transformed cells. This pp80 is postulated to be a tumor suppressor. Western analysis indicates that p80 is regulated at the level of synthesis with little or no p80 protein detectable in transformed JB6 cells. A cDNA clone of p80 has recently been isolated by screening a JB6 P- library with p80 antibody. This p80 cDNA, when used as a probe, detects a 5.2-kb RNA that progressively decreases during the progression from P- to transformed (Tx) phenotype. Recent studies on 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-inducible genes have focussed on those regulated by the trans-acting transcriptional factor, AP-1 (*jun* oncogene). The tumor promoters, TPA and epidermal growth factor, induce AP-1-regulated gene expression in P+ but not P- JB6 cells. This indicates that AP-1-regulated gene expession (1) may be required for tumor promoter-induced transformation; and (2) may be, in turn, controlled by activated *pro* genes found in P+ but not P- cells. The mechanism of differential trans-activation appears to involve differential induced levels of AP-1/*jun* protein.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Nancy H. Colburn	Chief, Cell Biology Section	LVC	NCI
Stephanie Simek	IRTA Fellow	LVC	NCI
Lori R. Bernstein	Biologist	LVC	NCI
Elia Ben-Ari	IRTA Fellow	LVC	NCI
Bonita M. Smith	Special Volunteer	LVC	NCI

Objectives:

To determine the required biochemical events that occur between the interaction of tumor promoters with the plasma membrane and the activation of intracellular effectors of neoplastic transformation. In the case of phorbol diester tumor promoters, a major aim is to identify promotion-relevant events that are closely coupled to phorbol ester receptor binding. Candidates for such second messengers or signal transduction events include protein kinase C (PKC)-catalyzed protein phosphorylation, and PKC-regulated trans-acting transcriptional factors AP-1, AP-2, etc. Tumor promoter-inducible AP-1-dependent gene expression will be analyzed for promotion relevance. An overall aim is to understand the nuclear gene regulation events triggered by activation of plasma membrane PKC. Finally, a PKC substrate of 80 kDa that progressively decreases during the progression from preneoplastic to tumor cell phenotype is being analyzed.

Methods Employed:

(1) Assay of calcium-dependent, phospholipid-dependent protein kinase (C-kinase or PKC) activity; (2) assay of the effects of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on rate of synthesis and phosphorylation of proteins in intact promotion-sensitive (P^+) or -resistant (P^-) cells; (3) immunoprecipitation and Western blotting with antisera to PKC substrates or other P^+-related proteins; (4) screening cDNA libraries using antibody or molecular probes; and (5) assay of a PKC-regulated trans-acting factor function such as AP-1-dependent gene expression.

Major Findings:

1. PKC substrate p80 shows progressive decrease in expression during progression from early preneoplastic to the neoplastic state. A PKC substrate of 80 kDa (p80) was found to be differentially phosphorylated in early preneoplastic (P^-), late preneoplastic (P^+) and transformed (Tx) JB6 mouse epidermal cells following exposure to TPA. The change observed was a progressive decrease in p80 phosphorylation from P^- to transformed phenotype. Recent Western analysis using antibody to p80 has revealed a progressive decrease in the amount of p80 expressed in P^-, P^+ and Tx cells, with the P^- cells containing the highest level and the transformed cells containing little or no detectable p80. TPA treatment causes a transient increase in the level of phosphorylation of p80 in P^- cells, reaching a sixfold maximum after 2

hours of TPA treatment, then returning to basal levels by 24 hours. In contrast, it has been shown that the synthesis of p80 in P^- cells remains unaffected by TPA treatment. Therefore, p80 appears to be regulated by TPA at the level of phosphorylation and not at the level of synthesis, but during preneoplastic progression, p80 is regulated at the level of synthesis. These results suggest that p80 may function as a suppressor of neoplastic transformation that is in some way switched off during the P^- to transformed cell progression.

2. Cloning of p80 from a P^- cDNA library. JB6 P^- cDNA was cloned into the Eco RI restriction site of the vector Lambda zap and the library was screened with p80 peptide antiserum. One positive clone containing a 2.4-kb insert was isolated and is currently being sequenced. The putative p80 clone was subcloned into the plasmid bluescript as a β-galactosidase fusion protein. The bacterial fusion protein was analyzed on a 10% sodium dodecyl sulfate-polyacrylamide gel and its size corresponded to an 80-kDa protein.

3. Hybridization of JB6 RNA to a putative p80 clone. The putative p80 clone was used as a probe in a hybridization reaction with total RNA isolated from P^- and transformed JB6 cells. A single 5.2-kb band was observed with P^- RNA, but little or no hybridization ($2 \pm 0.4\%$ of P^- level) appeared with RNA from transformed cells. This pattern was similar to what was observed for the differential expression of the p80 protein in these cells ($5 \pm 1.5\%$ of P^- level in Tx cells), thus suggesting regulation at the level of mRNA concentration.

4. AP-1/jun function is differentially induced in promotion-sensitive and promotion-resistant mouse epidermal JB6 cells. P^+ and P^- cells were transfected with a construct called 3X-AP1-CAT, which contains tandem AP-1 cis enhancer elements attached to a CAT reporter gene. P^+ cells display AP-1/jun-dependent trans-activation of CAT gene expression by TPA, epidermal growth factor, and serum (all transformation promoters in JB6 cells), whereas P^- cells are defective in this functional AP-1 response.

5. Introduction of jun expression constructs into P^- cells confers promotion sensitivity. A constitutive plasmid expression construct for the viral jun oncogene was generated and transfected into JB6 P^- and P^+ cells. The plasmid construct did not have complete transforming activity in either cell type. However, upon treatment with TPA, jun-transfected P^- cells displayed significant induction of anchorage-independent colonies in soft agar. An expression construct for the cellular jun oncogene showed similar P^+ activity in P^- cells. These data suggest that exposure of P^- cells to exogenous sources of AP-1/jun reconstitutes the promotion response to these cells, and suggest that the defect in promotion response may be due to defective induction of jun expression.

6. JB6 P^+ and P^- cells display differential synthesis of AP-1/jun protein. P^+ and P^- cells were labeled under steady state conditions with ^{35}S-methionine and immunoprecipitated with anti-AP-1 antisera before and after TPA treatment. These studies indicate a threefold induction of AP-1 protein in P^+ cells, while no induction was observed in P^- cells. The above data (#4-6) support the hypothesis that AP-1 is a required component in the signal transduction

pathway for the promotion of neoplastic transformation by TPA, and that the defective promotion response in P^- cells is attributable in part to a defect in TPA-induced AP-1/jun synthesis.

Publications:

Bernstein LR, Colburn NH. AP-1/jun function is differentially induced in promotion sensitive and resistant JB6 cells. Science 1989;244:566-70.

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Simek SL, Kligman D, Patel J, Colburn NH. Differential expression of an 80 kDA PKC substrate in preneoplastic and neoplastic mouse JB6 cells. Proc Natl Acad Sci USA (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05384-06 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 charecters or less Title must fit on one line between the borders.)
Genetic Analysis of Human Cellular Genes in Neoplastic Transformation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	Hector N. Seuanez	Visiting Scientist	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI
	Takis S. Papas	Chief	LMO	NCI
	Joost J. Oppenheim	Chief	LMI	NCI
	Stanley J. Cevario	Biologist	LVC	NCI

COOPERATING UNITS (if any) PRI,Fred.,MD(W.Modi,M.Dean); BRI,Fred.,MD(G.Vande Woude); CHB, NHLBI,Beth.,MD(N.Anagnou); Johns Hopkins Hosp.,Balt.,MD(B.Vogelstein); Rorer Biotech.,Hoesham,PA(M.Jaye); CEPH Center, Paris, France (J. Dausset); LDBA,NIDR (P. Killen); DBMB, NICHD, Beth.,MD (R. Klausner); Univ. Chicago, IL (R. Burke)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.1	0.5	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type Do not exceed the space provided)

The Section of Genetics has developed an efficient facility for gene mapping of human loci rapidly being discovered in the scientific community. Four methodologies are generally applied to new probes which become available in our laboratory or through collaborators. These include: (1) a panel of well-characterized rodent x human somatic cell hybrids, (2) in situ hybridization of molecular clones to human metaphase chromosomes, (3) detection of restriction fragment length polymorphisms by screens of human DNA's, and (4) linkage analysis in collaboration with the human family pedigrees administrated by the Centre D'Etude du Polymorphisme Humain-Human Polymorphism Study Center (CEPH) in Paris. We have collaborated with over 40 laboratories from the NIH and throughout the scientific community to map human loci. Loci mapped include proto-oncogenes, endogenous retroviral loci, growth factors, cell receptors, and several members of the immunoglobulin superfamily. The principle investigator serves in the human genome projects as an elected member of the Human Genome Organization (HUGO) as well as chairman of the International Committee of Comparative Gene Mapping.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
Hector N. Seuanez	Visiting Scientist	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
Takis S. Papas	Chief	LMO	NCI
Joost J. Oppenheim	Chief	LMI	NCI
Stanley J. Cevario	Biologist	LVC	NCI

Objectives:

1. The augmentation of the human gene map with loci that have direct or indirect connections to the processes of neoplastic transformation in man or other vertebrate model systems. The specific genes under study fall into six general groups: (1) cellular proto-oncogene loci, (2) genes which code for growth factors, (3) genes coding for receptors for growth factors and for retroviruses, (4) endogenous cellular DNA sequences homologous to retroviral RNA genomes, (5) chromosomal integration sites for chronic transforming retroviruses, and (6) restriction genes that delimit retroviral replication.

2. Genetic analysis of cooperative and sequential gene actions in the neoplastic processes and in virogene expression. This consideration involves application of the principles and techniques of microbial genetics to cultured mammalian cells.

3. The development of new approaches to the understanding of genetic control of carcinogenesis. This goal involves the identification and characterization of genetic targets (cellular genes) of carcinogenesis.

4. The resolution of the sequences and tissue specificities of distinct cellular genes in different human malignancies. Thus, neoplastic pathways of genetic networks can be dissected by genetic analysis in protocols reminiscent of dissection of metabolic enzyme pathways in early microbial studies.

Methods Employed:

The following techniques were employed: (1) somatic cell hybridization and various gene transfer procedures; (2) isoenzyme electrophoresis and histochemical development on starch, acrylamide and cellulose acetate; (3) cytological chromosome banding procedures (G and Q, G-11) for specific chromosome identification; (4) virological procedures including radio- immunoassay (RIA), reverse transcriptase assay and viral cloning; (5) immunological assays, including RIA, cytotoxicity, fluorescent antibody procedures and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques, including cDNA transcription in vitro, solution hybridization, visualization of restricted DNA by the techniques of Southern following agarose electrophoresis, and molecular cloning of eukaryotic genes; (7) in situ

hybridization to metaphase chromosomes; and (8) reverse fragment length polymorphism (RFLP) linkage analysis of human pedigrees and populations.

Major Findings:

1. The proto-oncogene family in man. Transforming genes, or proto-oncogenes, represent a class of conserved cellular genes that may play an important role in tumorigenesis. They were initially described as transduced RNA segments in transforming retrovirus genomes. They have also been discovered by focus induction after transfection of mouse NIH 3T3 cells with genomic DNA extracted from human tumors.

In collaboration with laboratories within and outside the NIH, we have mapped over one-third of the proto-oncogene loci in man. Several have included pseudogenes (ras and raf). Others have shown evidence of gene fusion (ets, met-tpr, trk, fgr). Separation of these components using hybrid panels has resolved the fusion-fission events in the evolutionary history of the oncogenes. (See Table 1 for a list of genes mapped in the Section of Genetics.)

2. Cloning and chromosomal mapping of the human T-cell receptor zeta:chain. The T-cell antigen receptor (TCR) is a multisubunit receptor complex specific to T-cells subserving both antigen recognition and signal transduction functions. The ζ (zeta) chain of the TCR is a component of all surface receptor complexes. This chain was first identified in murine T-cells by virtue of the fact that it co-immune precipitates with the TCR complex using antibodies directed against either the clone-specific subunits or invariant CD3 subunits of the receptor. Recently we have isolated a cDNA encoding the murine ζ. Using this as a probe, we have now isolated cDNA's encoding the human ζ. Sequence analysis of cDNA's encoding human and murine ζ reveals that it is a highly conserved protein. In addition to amino acid homology, there is remarkable interspecies conservation in the nucleotide sequence of the 5′ and 3′ untranslated regions of the ζ mRNA. The previously characterized invariant δ, ϵ, and γ chains of the TCR, referred to as the CD3 complex, share significant sequence and structural homology with each other and are all located within 300 kb of each other on human chromosome 11(11q23). Zeta has no sequence similar to the CD3 chains, and the localization of the human ζ gene to the centromeric region of chromosome 1 underscores the fact that it is a distinct genetic component of the TCR.

3. Genetic characterization of the GLI-Kruppel family of human genes. Previous characterization of GLI, a gene found to be amplified and expressed in a subset of human brain tumors, revealed the presence of five tandem zinc fingers related to those of Kruppel (Kr), a Drosophila segmentation gene of the gap class. We have used the GLI cDNA as a molecular probe to isolate related sequences from the human genome. Partial characterization of six related loci, including sequence determination, expression studies, and chromosome localization, revealed that each locus could encode a separate finger protein. The predicted proteins all had similar H-C links, i.e., a conserved stretch of nine amino acids connecting the C-terminal histidine of one finger to the N-terminal cysteine of the next. On the basis of amino acid sequence and intron-exon organization, the genes could be placed into one of

two subgroups: the GLI subgroup (with the consensus finger amino acid sequence [Y/F]XCX$_3$GCX$_3$[F/Y]X$_5$LX$_2$HX$_{3-4}$H[T/S]GEKP) or the Kr subgroup (with the consensus finger amino acid sequence [Y/F]XCX$_2$CX$_3$FX$_5$LX$_2$HXRXHTGEKP). Unlike GLI or Kr, most of the newly isolated genes were expressed in many adult tissues. The predicted proteins probably control the expression of other genes and, by analogy with Kr and GLI, may be important in human development, tissue-specific differentiation, or neoplasia.

4. Human erg proto-oncogene maps to chromosome 21q22, near a specific 8:21 translocation of acute myelogenous leukemia (AML). There is accumulating evidence to support that genes on chromosome 21 play an important role in the development of pathologies associated with leukemia, Down's syndrome, and Alzheimer's disease. We have previously described erg, a human gene related to the ets oncogene. In this study, we have regionally assigned the erg gene to chromosome 21q22.3 by using somatic cell hybrids and in situ hybridization analysis. In light of this chromosome assignment, the relationship of erg to the 21q translocation breakpoint characteristic of AML was considered. By using a DNA probe that is specific for the erg gene, a panel of rodent-human cell hybrids was analyzed by the Southern technique to study specific chromosome translocations occurring in acute myeloblastic leukemia. The erg gene was found to translocate from chromosome 21 to 8 in the t(8;21)(q22;q22), a non-random translocation found in patients with AML of the subgroup M2 (AML-M2). The localization of the erg gene to chromosome 21q22 raises the possibility that this gene may be involved in the pathogenesis of AML-M2.

5. Human interleukin 1α (IL-1α) gene is located on chromosome 2q12-21. The IL-1α gene was assigned to chromosome 2 using Southern transfer analysis of human-rodent somatic cell hybrid DNA's. The gene was regionally localized to 2q12-21 using in situ hybridization to metaphase chromosomes. These results indicate that the IL-1α gene maps to the same general region on the long arm of chromosome 2 as the IL-1β gene, which has been previously assigned.

6. A human homologue to a murine chromosomal locus which is a preferred target for integration by leukemogenic murine leukemia virus. The Moloney leukemia virus integration 2 (Mlvi-2) locus represents a common region for proviral integration and a putative oncogene involved in the induction of thymic lymphomas in rodents. The human homologue of the Mlvi-2 locus has been cloned, and studies have been initiated to determine its possible role in the induction and progression of human neoplasms. In this study we used a panel of human x rodent somatic cell hybrids and in situ hybridization to metaphase chromosomes to map Mlvi-2 to the short arm of the human chromosome 5, band p14.

7. The human protamine/locus is on human chromosome 16 and its mouse homologue is on mouse chromosome 16. The protamines are small arginine-rich nuclear proteins that replace histones and transition proteins late in the haploid phase of spermatogenesis in mammals. The two mouse genes encoding protamines, Prm-1 and Prm-2, have been molecularly cloned and mapped to mouse chromosome 16 (MMU 16). A cDNA clone of mouse Prm-1 which hybridized to the corresponding human gene was utilized to analyze a panel of somatic cell hybrids made between human lymphoblasts and the E36 hamster cell line. The

human gene, which we have designated PRM1, was syntenic with human chromosome 16 (HSA 16) and discordant with all other human chromosomes. Linkage analysis in the mouse was accomplished using the backcross (Czech II X BALB/cPt) X Czech II to map Prm-1 and Prm-2 to a position near the 5' terminus of MMU 16. No recombination between Prm-1 and Prm-2 was observed among 89 progeny of the Czech II by BALB/c cross nor among 94 progeny of the backcross (CBA/J X BALB/cJ) X BALB/cJ, demonstrating that the two loci are separated by less than 1.6 centiMorgans (cM) on MMU 16. This tight linkage may be of functional significance, since Prm-1 and Prm-2 are among a limited number of genes known to be expressed post-meiotically in male haploid germ cells.

8. Glutamase gene homologues map to human chromosome 2, to mouse chromosome 1, and to rat chromosome 9. A rat cDNA clone encoding a portion of phosphate-activated glutaminase was used to identify DNA RFLP's in sets of somatic cell hybrids and between wild-derived and inbred strains of mice. Segregation of rat and mouse chromosomes among somatic cell hybrids indicated assignment to rat chromosome 9 and mouse chromosome 1. Analysis of chromosome 1 alleles for several genes in an interspecific cross between Mus spretus and C3H/HeJ-gld/gld mice indicate that glutaminase can be positioned within 5.5 ± 2.0 cM proximal to Ctla-4. Similarly, human-hamster somatic cell hybrids were examined for RFLP's and four human EcoRI restriction fragments were found to hybridize with the rat glutaminase probe. Two of these restriction fragments cosegregated and mapped to human chromosome 2 in a region that is syntenic with mouse chromosome 1 and rat chromosome 9.

Publications:

Anagnou NP, Economu-Pachnis A, O'Brien SJ, Modi WS, Nienhuis AW, Tsichlis PN. The human homologue of the Moloney leukemia virus integration 2 locus (Mlvi-2) maps to band p14 of chromosome 5. Genomics (In Press).

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Ruppert JM, Kinzler KW, Wong AJ, Bigner SH, Kai FT, Law ML, Seuànez H, O'Brien SJ, Vogelstein B. The GLI-Kruppel family of human genes. Mol Cell Biol 1988;8:3104-13.

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Weissman AM, Hou D, Orloff DG, Modi WS, Seuànez HN, O'Brien SJ, Klausner RD. Molecular cloning and chromosomal localization of the human T-cell receptor Z chain: distinction from the molecular CD3 complex. Proc Natl Acad Sci USA 1988;85:9709-13.

Gene Abbreviation	Gene Name	Location	Collaborator	Citation
ERG	ets-related gene	21q22	S. Reddy, T. Papas	Rao et al., In press
IL-1α	interleukin 1 alpha	2q12-21	K. Matsushima, J. Oppenheim	Modi et al., 1988
MDNCF	monocyte derived neutrophil chemotactic factor	4q13-21	K. Matsushima	In preparation
TCR-ZETA	T-cell receptor, zeta chain	1p22-q21	A. Weissman, R. Klausner	Weissman, et al., In press
OVC	ovarian carcinoma	9p24	D. Blair	In preparation
λHLM-25	endogenous retrovirus	5q33-35	N. Anagnou	Anagnou et al., Submitted
ENDO	endonexin II	4q28-31	M. Jaye	In preparation
CGA	chromogranin A	14q32	M. Levine	Modi et al., Submitted
G-PROT	GTP binding protein beta III	12p13	M. Levine	In preparation
HMR	acetylcholine muscarinic receptors	1,7,11,11,15	T. Bonner	In preparation
GLUD	glutamate dehydrogenase	10q11 and xq26-28	N. Anagnou	In preparation
TPR	translocated promoter region	1q27-32	M. Park, G. Vande Woude	In preparation
MLVI-2	Moloney leukemia virus integration	5p14	N. Anagnou	Anagnou et al., 1988
GLI-K	glioma gene family	1,2,7,8,19,19	B. Vogelstein	Ruppert et al., 1988
NS-1	oncogene	In progress	S. Morse	In preparation
ACT-1	immune activation gene	In progress	W. Leonard	In preparation
CALB	calbindin	In progress	S. Christakos	In preparation
LPCO	lipocortin	In progress	L. Burns	In preparation
COLOEC	coloectrin	In progress	L. Burns	In preparation
CALP	calpactin	In progress	L. Burns	In preparation
GLU-1,2	glutaminase genes	2	B. Mock	Mock et al., In Press
ACT-2	immune activation gene 2	In progress	W. Leonard	In preparation
ADA2	alcohol dehydrogenase	4	D. Goldman	Goldman et al., Submitted
MCF	monocyte chemostatic factor	In progress	N. Yuhki	In preparation
SAA	serum amyloid	11	G. Sack	Sack et al., 1988

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05385-06 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Molecular Genetic Analysis of Feline Cellular Genes: A Comparative Approach

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	David Derse	Senior Staff Fellow	LVC	NCI
	Naoya Yuhki	Visiting Associate	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary A. Eichelberger	Microbiologist	LVC	NCI

COOPERATING UNITS (if any)
PRI, Frederick, MD (D.A. Gilbert, W.S. Modi); H&W Cytogenetics Services, Inc., Lovettsville, VA (W.G. Nash); Univ. of CA, San Diego, CA (J.S. O'Brien); NIAID, NIH, Bethesda, MD (C. Kozak); Washington State Univ., Pullman, WA (J. Evermann)

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SECTION
Genetics Section

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TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.7	0.9	0.8

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Construction of a gene map of the domestic cat (Felis catus) employing a well-characterized panel of rodent x cat somatic cell hybrids is continued. The present map consists of over 70 biochemical (isozyme or DNA clone) loci. A remarkable extent of linkage homology between the feline and human maps was discovered that was three to four times more conserved than the mouse-to-human genetic synteny (linkage homology). Nearly 35% of the human cytological map can be aligned, band-for-band, with syntenically homologous feline chromosomes. This degree of linkage homology was used to estimate chromosomal location of feline homologs of proto-oncogenes and to test for transposition of the proto-oncogene family during the over 80 million years of evolution which has elapsed since man and cat shared a common ancestor. The organization of three distinct endogenous retroviral families was studied and found to resemble endogenous retroviral families in other mammalian species, including man. Genetic loci, which encode a series of lysosomal enzymes involved in feline models of human neurological storage diseases, have been localized. A molecular phylogeny of the Felidae family has been derived based upon three methodologies, and a cytogenetic description of Felidae evolution was developed.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
David Derse	Senior Staff Fellow	LVC	NCI
Naoya Yuhki	Visiting Associate	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI

Objectives:

(1) The development and expansion of the genetic map of the domestic cat (Felis catus) with particular emphasis on molecular genetic loci involved in neoplastic transformation. (2) The understanding of the genomic and developmental organization of feline loci involved in cancer. (3) The description of the comparative structure of the cat genome relative to other felids, to other carnivores, and to other mammals, specifically, mouse and man. (4) The development of the gene delivery technologies for treatment of feline models of human inborn errors.

Methods Employed:

The following techniques were employed: (1) somatic cell hybridization and various gene transfer procedures; (2) isoenzyme electrophoresis and histochemical development on starch, acrylamide, and cellulose acetate; (3) cytological chromosome banding procedures (G and Q, G-11) for specific chromosome identification; (4) virological procedures, including radio-immunoassay (RIA), reverse transcriptase assay, and viral cloning; (5) immunological assays, including RIA, cytotoxicity, fluorescent antibody procedures, immunoprecipitation, microcomplement fixation, and monoclonal antibody preparation in murine hybridomas; (6) molecular biology techniques, including cDNA transcription in vitro, molecular cloning, gene splicing, DNA and RNA blotting; and (7) in situ DNA hybridization to metaphase chromosomes.

Major Findings:

1. Constructing the gene map of the cat: Extensive conservation of linkage arrangement to the human genetic map. A research emphasis on the construction of the genetic map of the cat has been a major focus in our laboratory for several years. The cat gene map now consists of over 70 loci, including some 30 proto-oncogene loci. With the exception of a preliminary synteny map for the domestic dog, the only other carnivore gene-chromosome map was derived by the Russian geneticist, O. L. Serov and his colleagues for the American mink (Mustela vision). In nearly all cases where they could be compared, the mink and cat linkages agree, thereby confirming the cytological indication of homology. When we first compared linkage maps of cat and man, we were struck by the high degree of syntenic homology which existed between the two species, especially when we considered that cat and man were in different mammalian

orders. In most cases, syntenic groups located on human chromosome arms were also syntenic in cats and in the case of at least five human chromosomes (1, 6, 11, 12, and X), gene homologs from both arms are also syntenic in cats, suggesting conservation of large portions of these chromosomes. By contrast, the mouse gene map is three to four times more rearranged, compared with man, than is that of the cat.

The striking linkage homology between cat and man, combined with certain advantageous cytological characteristics of primate and feline chromosomal evolution, prompted us to search for cytological homology between syntenically homologous chromosomes in the two species. High-resolution, G-banded preparations of homologous chromosomes were carefully examined, and several regions of band-for-band homology were identified (HSA1p:FCAC1, HSA2p:FCAA3, HSA2q:FCAC1, HSA11:FCAD1, and HSA12:FCAB4). In all, we could align between 30 and 35% of the human karyotype band-for-band with the feline karyotype despite the passage of over 80 million years since these species shared a common ancestor.

2. Conservation of proto-oncogene chromosomal location in the cat. Proto-oncogenes represent a class of evolutionarily conserved cellular genes which may play an important role in tumorigenesis. The physiological function of the majority of normal proto-oncogenes has only just begun to be clarified, although the fact that some encode growth factors (sis) or receptors (erbB, fms) and that all are precisely regulated during development suggests a critical role for these genes. Because of the prevalence of feline leukemia virus (FeLV) in pet cats, this species has been the source of more retroviral oncogene isolations than any other mammal. The identification of homologous chromosomal segments between cat and man, discussed above, combined with the accumulated genetic and molecular information of the vertebrate oncogenes, provide a good opportunity to search for transposition of cellular genes during the more than 80 million years since primates and carnivores shared a common ancestor. Oncogenes are prime candidates for such a consideration because they are the best-known targets for retroviral recombination. Approximately 30 oncogenes and growth factor loci have been chromosomally assigned in the cat. Nearly all of these fall in chromosomal linkage groups which would be predicted by the human-cat syntenic map.

3. Genetic loci for feline lysosomal enzymes: Model for human storage diseases. The lysosomal storage diseases of man are fatal disorders of the nervous system resulting from inherited defects in catabolism of gangliosides and other complex glycolipids and glycoproteins. The specific enzyme defects responsible for a variety of human lysosomal storage diseases have been identified and characterized, and more recently, the normal genes coding for many lysosomal enzymes have been isolated. Several of these diseases have model systems in the domestic cat which render the species of potential use for developing gene therapy. An initial step in this project was to employ the feline mapping panel to genetically map the feline genetic loci which encode the affected enzymes. A panel of 42 rodent x cat somatic cell hybrids has been used to assign eight structural genes for lysosomal enzymes to specific chromosomes in the domestic cat. The assignments include alpha-glucosidase (GANAB) to chromosome D1, alpha-galactosidase (GLA) to the X chromosome, beta-galactosidase-1 (GLB1) to chromosome B3, beta-glucuronidase (GUSB) to chromosome E3, alpha-mannosidase A (MANA) to chromosome B3,

alpha-L-fucosidase (FUCA) to chromosome C1, hexose-aminidase A (HEXA) to chromosome B3, and alpha-L-iduronidase (IDUA) to chromosome D4. In all cases, the feline lysosomal enzyme genes were located in linkage groups which were syntenic with their homologous position in the human gene map. These assignments expand the genetic map of the cat and reaffirm the extensive syntenic homology between the chromosome maps of man and cat.

4. Development and characterization of monoclonal antibodies reacting with cell surface markers on feline lymphomas. The immunological and cytochemical phenotypes of five primary feline lymphomas and six feline lymphoma lines are reported. Thymic lymphomas induced by the Rickard strain of FeLV (FeLV-R) are of prothymocyte or (immature) cortical thymocyte origin, as these express terminal deoxynucleotidyl transferase, the guinea pig erythrocyte rosette receptor, Ia antigens, partial cortisone sensitivity, and nonspecific esterase. Lymphomas associated with other strains of FeLV form rosettes with guinea pig erythrocytes, frequently have Ia antigens and cytoplasmic nonspecific esterase, and probably originate from helper T-cells, monocyte/macrophages, or null cells. These data belie previous conclusions that FeLV leukemogenesis is restricted to mature T-cells; rather, the considerable heterogeneity in the surface and cytochemical phenotype of feline lymphomas probably reflects transformation of multipotent lymphoid or monocytoid precursors in the bone marrow by FeLV.

5. Molecular and genetic characterization of feline and canine satellite DNA sequences. Cloned satellite DNAs which hybridize primarily to C-band positive regions of felid and canid chromosomes were used to probe the structures of satellite loci in the genomes of 16 species of felids and 15 species of canids. Southern blot and quantitative dot blot experiments demonstrated that satellite loci within the great cats, genus Panthera are quite variable in regard to amount, and somewhat variable in regard to restriction patterns. Satellite loci within the canids appeared more uniform in regard to both amounts and restriction patterns, although some canid species did differ significantly from the consensus pattern in both respects. Even though intrafamilial satellite restriction patterns were generally quite similar, every species could be shown to have a unique and characteristic pattern.

In situ hybridization was carried out using cloned satellite DNAs from the domestic cat and domestic dog as probes to metaphase chromosomes from 12 species of felids and 10 species of canids. Autoradiographic silver grains along metaphase chromosomes were counted and are considered with regard to: the mean number of grains per cell in each species, their chromosomal location, and their presence or absence on specific autosomes or sex chromosomes where known. Among the felids and canids there was a 7.6- or 8.9-fold statistically significant difference, respectively, in the mean number of grains per cell between the species having the minimum and maximum values. Among the felids, most grains occurred on the tolomeres of D and E group chromosomes, although departures from this general pattern exist. For example, the Asian golden cat and the Bornean bay cat showed substantial labeling at the centromeric region of chromosome A1 and a number of species showed some labeling at the short arm telomeres of B group chromosomes. Among the canids, about 90% of all grains were found to occur at autosomal centromeres and grains were found to be absent from the sex chromosomes.

Grain distribution and the presence of constitutive heterochromatin, as evidenced by C-banding, are coincident in certain cases but not in others.

6. Isolation and characterization of a pathological feline infectious peritonitis virus (FIPV) from a captive cheetah (Acinonyx jubatus). A coronavirus which was isolated from a cheetah (Acinonyx jubatus) that succumbed to FIP was characterized in vitro. The virus was determined to be highly cell-associated with Crandell feline kidney (CrFK) cells and was routinely maintained as a persistent infection (CrFK 83-4497). The cheetah coronavirus was compared with other members of the feline coronavirus group, including the feline enteric coronavirus (FECV) 79-1683 and the FIPVs, 79-1146, and UCD-1. The cheetah coronavirus was demonstrated to have a restricted host-cell range with limited cytopathic effect. Immunofluorescence with antisera to FIPV UCD-1 revealed the concentration of viral antigens in the perinuclear region of cells infected with the cheetah coronavirus. Ultrastructural studies of the cheetah coronavirus indicated a limited number of viral particles within cytoplasmic vesicles and few mature virus particles at the cell surface. This was in contrast to electron microscopy results of FECV 79-1683 and FIPV 79-1146, which had numerous virus particles within the cytoplasmic vesicles, as well as at the cell surface. The cheetah coronavirus was tentatively placed in the feline coronavirus family based upon its antigenic reactivity by immunofluorescence; however, the possibility that it represents a unique coronavirus of cheetahs should not be dismissed without further analyses at the host and genomic levels.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05389-06 LVC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Reproductive Strategies in Animal Species Emphasizing Developmental Biology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	David E. Wildt	Special Volunteer	LVC	NCI
	Leslie Johnston	IRTA Fellow	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI

COOPERATING UNITS *(if any)*

Dept. of Animal Health, Natl. Zool. Park, Wash., DC (A.M. Miller, J.G. Howard, M.C. Schiewe, S. Hurlburt, M. Bush); Veterinary Resources Branch, DRS, NIH, Bethesda, MD (P.M. Schmidt)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.7	1.3	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The purpose of this project is to study and define the biological factors, including the influence of genetics, on reproductive function of mammalian species. Particular emphasis is placed on gamete interaction and developmental biology through the blastocyst stage of embryo development. Findings are relevant to fundamental studies of fertilization mechanisms, the propagation of genetically valuable laboratory animals and endangered species, and in developmental studies which eventually will allow delivering molecularly cloned genes into early-staged pre-implantation embryos. Areas of effort in the female primarily focus on (1) the influence of exogenous gonadotropins on ovarian follicular recruitment and oocyte integrity and maturation; (2) in vitro fertilization and embryo transfer; and (3) oocyte rescue, maturation in vitro, and the development of gene delivery techniques into embryos which will allow studying the mechanisms associated with transformation and inborn errors in early development. Efforts are complemented by multidisciplinary research in male reproduction, particularly in the fields of sperm cell integrity and function tested by in vitro fertilization systems using both homologous and heterologous oocytes. Current emphasis is applied to the gametes and embryos of the domestic cat with comparative studies conducted in a variety of nondomesticated Felidae species. Progress to date has allowed (1) routine collection of structurally normal intrafollicular oocytes from gonadotropin-treated cats and the production of embryos by in vitro fertilization; (2) successful and routine in vitro culture of in vitro fertilized cat embryos to the morula stage of development; (3) production of live, healthy young from in vitro fertilized and surgically transferred embryos; (4) in vitro maturation of immature, antral oocytes and the fertilization and development of these oocytes to the 16-cell embryonic stage in vitro; (5) development of heterologous and homologous oocyte in vitro fertilization assays which allow determining the impact of teratospermia on the fertilization event; and (6) successful recovery and in vitro fertilization of follicular oocytes from two nondomestic species of Felidae.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
David E. Wildt	Special Volunteer	LVC	NCI
Leslie Johnston	IRTA Fellow	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI

Objectives:

The specific objectives of these studies are (1) to understand the developmental biology of the domestic cat; and (2) to increase and integrate the reproductive-genetic data base, thereby permitting improved propagation, embryological manipulation and gene transfer technology in the cat and related species.

Methods Employed:

The following techniques were used: (1) hormonal induction of ovarian activity; (2) laparoscopic recovery of ovarian follicular oocytes; (3) electroejaculation and laboratory processing for sperm recovery and induction of capacitation; (4) light and electron microscopy for evaluation of spermatozoal function and integrity; (5) in vitro oocyte maturation, fertilization, and embryo culture; and (6) surgical procedures for embryo transfer.

Major Findings:

1. In vitro fertilization (IVF) of follicular oocytes in the Felidae. Little is known about the fertilization processes leading to embryo development in cats. IVF offers a valuable approach for studying fertilization and early embryogenesis as well as the species-specific variables which affect gamete fusion. This project focuses on examining the basic factors allowing routine production of cat embryos via IVF, a strategy which eventually will permit manipulating the felid genome. In FY 89, studies emphasized the basic processes of fertilization, including the influence of exogenous hormones on oocyte integrity and subsequent fertilizability.

The effect of the interval between pregnant mares serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG, required for oocyte maturation) on follicular recruitment, oocyte maturation, and fertilizability in vitro was examined. Cats with inactive ovaries were given PMSG followed 80 or 84 hours (h) later by hCG. Prolonging the PMSG/hCG interval 4 h increased the number of follicles available and total mature oocytes (based on cumulus cell mass and corona radiata expansion) recovered by 37% and 61%, respectively. Number of mature oocytes recovered and cleavage rate were greater for the 84-h (16.3% and 81%, respectively) compared to the 80-h (6.5% and 55%, respectively) treatment group, likely as a result of enhanced intrafollicular oocyte maturation.

Based on the domestic cat system, IVF studies were conducted in the taxo-taxonomically-related leopard cat (Felis bengalensis) receiving PMSG followed 80 or 84 h later by hCG. Mean number of follicles present and oocytes collected did not differ between gonadotropin interval groups. However, the proportion of mature leopard cat oocytes fertilized in vitro, as determined by embryonic cleavage, was increased by extending the PMSG to hCG interval from 80 (17.5%) to 84 (52.4%) h. Compared to the domestic cat, the ovaries of the leopard cat were less responsive to treatment, but nonetheless were readily capable of fertilizing in vitro. Studies were extended to the puma (Felis concolor), a species producing a high incidence of teratospermia. Seven of 8 pumas responded to gonadotropin treatment and 140 eggs were recovered from 145 follicles (20.0 oocytes/female). Overall IVF rate was 43.5% despite using inseminants containing 82% to 99% pleiomorphic spermatozoa. The successful IVF of leopard cat and puma follicular oocytes using a system developed in the domestic cat suggests that this approach may be valuable for enhancing the propagation of rare Felidae.

Because of the vast potential of this technology for understanding gamete interaction in carnivores and manipulating the genome of the pre-implantation embryo, plans for FY 90 will focus on evaluating (a) early embryo development through chromosomal analysis of embryos and hormonal evaluation of donor females; and (b) the influence of culture medium, temperature, gas phase, and gamete aging on fertilization and embryo culture.

2. In vitro fertilization of in vitro-matured follicular oocytes in the Felidae. In vitro maturation of oocytes followed by IVF and embryo transfer has resulted in the birth of young in the mouse, rat, sheep, and cow. The routine production of cat embryos from follicular oocytes would allow the domestic cat to be used more readily in developmental biology research, especially for examining several naturally occurring inborn errors of metabolism. The capability of maturing and then fertilizing antral oocytes also would provide a valuable approach for rescuing genetic material from rare species of Felidae. Studies in FY 89 defined the conditions necessary for oocyte maturation and subsequent IVF in the domestic cat.

Oocytes, collected from ovaries removed at ovariohysterectomy, were cultured and evaluated for nuclear maturation by analyzing chromosomal spreads. Oocytes achieved metaphase II after intervals of 40 to 48 h of in vitro incubation. The incidence of maturation was enhanced when oocytes were recovered from inactive or follicular stage donors compared to those recovered from luteal phase or pregnant cats. The proportion of oocytes successfully maturing in vitro in medium containing no hormone supplementation was less than counterparts cultured in follicle stimulating hormone (FSH) only or FSH plus luteinizing hormone. The efficiency of maturation was not influenced by delaying recovery of oocytes from antral follicles (from 8 h to 32 h). Approximately 36% of the in vitro-matured oocytes co-cultured with spermatozoa demonstrated evidence of fertilization. Under current optimal conditions, 50% to 60% of oocytes collected directly from antral follicles will mature in vitro and, after insemination, approximately 30% of these can be expected to cleave to the 2-cell stage of development.

In FY 90, these studies will be expanded by more detailed evaluation of the culture conditions necessary to promote in vitro maturation of immature follicular oocytes. Particular emphasis will focus on increasing the successful IVF rate of matured oocytes to 50% or greater.

3. Oocyte penetration assays as functional tests of spermatozoal viability. Many species of Felidae produce a high incidence of morphologically abnormal spermatozoa/ejaculate (teratospermia). Limited data are available on the processes of gamete interaction in carnivores, including the influence of seminal quality on ovum penetration and capacitation. In FY 89, progress was made in developing in vitro procedures for studying sperm functionality. The ability of spermatozoa to bind and penetrate zona-free hamster ova and zona-intact domestic cat oocytes in vitro was compared using ejaculates collected from normospermic (>60% structurally normal spermatozoa/ejaculate) and teratospermic (<40% normal spermatozoa/ejaculate) domestic cats.

Spermatozoa from both cat populations were capable of binding to and penetrating zona-pellucida-free hamster ova. High percentages of structurally normal spermatozoa were bound to ova following co-incubation, regardless of the morphological forms in the inseminant. Mean percent normal spermatozoa bound to oocytes from teratospermic males were no different from similarly treated aliquots from normospermic males. However, the overall penetration of hamster ova by normospermic ejaculates was superior to that observed using teratospermic ejaculates. Spermatozoa from teratospermic domestic cats were capable of binding and penetrating zona-intact cat oocytes; however, the number of bound spermatozoa/ovum was fivefold less in the males. These results indicated that teratospermia in domestic cats has a detrimental impact on gamete interaction and penetration of zona-free heterologous ova and zona-intact homologous ova. The effect was not totally dependent on the absolute numbers of normal spermatozoa in either in vitro system, but rather appeared related to an as yet unidentified factor(s) characteristic of teratospermic ejaculates.

In FY 90, comparative studies will be extended to detailing sperm functionality in the context of the fertilization event in the domestic as well as nondomestic cat. These studies will be complemented by detailed physicochemical evaluations of the domestic cat ejaculate, including assessments of osmolality.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05414-06 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Characterization of Retroviruses (Type-D and SIVs) Isolated from Primates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Raoul E. Benveniste	Medical Officer	LVC	NCI
Others:	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
	David Derse	Senior Staff Fellow	LVC	NCI

COOPERATING UNITS (if any) Univ. of Washington, Seattle, WA (W. Morton, C.-C. Tsai); Bionetics Research, Inc., Frederick, MD (L. Henderson, S. Oroszlan); USAMRIID, Frederick, MD (P.B. Jahrling); Program Resources, Inc., Frederick, MD (M. Gonda); Biotech Research, Rockville, MD (T. Li).

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Immunogenetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.3	0.7	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Three primate lentiviruses (simian immunodeficiency viruses, SIV) have been isolated from a macaque (M. nemestrina) housed at the University of Washington Primate Center (SIV/Mne), from a wild-caught mangabey (SIV/Cat) and from a colony-housed African green monkey (SIV/Cae). These three isolates can be readily distinguished from each other based on their cell tropism in culture and the immunological cross-reactivity of their viral proteins. SIV/Mne and SIV/Cat grow well in human and primate macrophages. The gag and env proteins of these two viruses have been purified and the amino acid sequences determined. SIV/Mne has been inoculated intravenously into three species of macaques (17 animals); virus was isolated from all the macaques who subsequently died at 15 to 120 weeks (mean 80 weeks) with various manifestations of immune deficiency. A single-cell clone (clone E11S) and a molecular clone (clone 5) have been obtained from SIV/Mne. Clone E11S contains two integrated proviruses per cell and has large amounts of gp120 envelope protein associated with the virus even after sucrose gradient purification. The molecular clone has been completely sequenced and is 95% homologous to SIV/Mac isolated from another macaque, 82% identical to human immunodeficiency virus-2 (HIV-2), and 42% to HIV-1. This full-length clone is infectious in vitro and in vivo. One of two macaques infected with the molecular clone has a marked decrease in CD4+ peripheral blood lymphocytes 60 weeks after inoculation.

HIV-1 readily forms syncytia after infecting a variety of cell lines in culture. None of these cell lines can be infected with SIV's. We have identified an Epstein Barr virus-transformed human cell line that readily forms syncytia 48 hours after infection with all three SIV isolates. This property has led to the development of a rapid in vitro assay to test for virus neutralizing activity in primate sera.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Raoul E. Benveniste	Medical Officer	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
David Derse	Senior Staff Fellow	LVC	NCI

Objectives:

To characterize primate retroviruses, with emphasis on the type-D and lentivirus (simian immunodeficiency virus, SIV) classes. To determine, by molecular hybridization and immunological techniques, the homology between these viruses and other primate retroviruses.

New isolates will be characterized by examining their host range for various cells in vitro, by obtaining molecular clones, restriction enzyme maps, and DNA sequence data. In addition, viral proteins will be purified, amino acid sequences determined, and antisera to the individual proteins raised in rabbits in order to develop specific immunological reagents and to examine the extent of antigenic and molecular similarities to human AIDS viral isolates. The pathogenicity of these isolates in various primate species will be determined in order to develop a suitable animal model for AIDS. The effect of various genes on pathogenicity will be determined by performing site-directed mutagenesis and rechallenging primates with these new variants.

To determine the prevalence of these primate viruses in various primate colonies and in feral populations by examining sera for the presence of cross-reactive antibodies and peripheral blood lymphocytes (PBL's) for the presence of viruses.

Methods Employed:

Virus isolation was attempted using cell lines that have previously been employed for the isolation of other primate and human retroviruses. Fresh tumors, whole blood, or sera obtained from primates were cocultivated with various cells and the supernatant assayed at intervals for reverse transcriptase activity. Cloned retroviral DNA was used as a probe for detection of related DNA sequences in primates. Viral proteins were purified by high pressure liquid chromatography (HPLC). Antigens and antibodies were detected by radioimmunoassays, ELISA assays, and by Western immunoblot techniques.

Major Findings:

1. Isolation and molecular characterization of lentiviruses (SIV class) from primates. SIV/Mne was isolated from a pig-tailed macaque (M. nemestrina) that had died with malignant lymphoma at the University of Washington Regional Primate Research Center. Additional SIV's have been isolated on human PBL's after cocultivating frozen spleen from a wild-caught mangabey captured in 1974, and on the CEM cell line by cocultivating fresh PBL's obtained from an

African green monkey housed at the USAMRIID colony. These isolates are named SIV/Cat (for Cercocebus atys) and SIV/Cae (for Cercopithecus aethiops), respectively.

These three isolates can be distinguished by their tropism in cell culture; SIV/Cat and SIV/Mne replicate readily in human monocyte-macrophage cultures. The gag and env proteins of SIV/Cat and SIV/Mne have been purified by HPLC and the amino acid sequences determined and compared to that of other primate lentiviruses. SIV/Cat and SIV/Mne are 85% related to each other and both are approximately 82% related to HIV-2 and 42% to HIV-1. Polyclonal rabbit antisera have been obtained from these purified viral proteins and used in radioimmunoassays and Western blots. These viruses can be readily distinguished on the basis of the immunological cross-reactivity of their viral proteins.

2. Pathogenicity of SIV/Mne. End-point diluted SIV/Mne, grown in either human PBL's or the HuT-78 cell line, has been inoculated intravenously into 17 macaques belonging to three species (M. mulatta, M. nemestrina, and M. fascicularis), and into 2 baboons (P. cynocephalus). Virus could be recovered from all 17 macaques starting 2 weeks after inoculation; and intermittently but continuously thereafter. Sixteen animals seroconverted and died at 43 to 120 weeks after inoculation (mean=80 weeks) with a marked decrease in $CD4^+$ cells and various manifestations of immune deficiency. The remaining animal did not seroconvert and died at 15 weeks with the same symptoms. The baboons did not become infected, did not seroconvert, and remained healthy until terminated from the study at 1 year.

3. Characterization of biological and molecular clones of SIV/Mne. Various single-cell clones of HuT-78 cells infected with end-point diluted SIV/Mne were obtained on microtiter plates seeded with a feeder layer of sheep choroid plexus cells. Clone E11S contains two integrated proviruses per cell and has large amounts of env gp120 even after sucrose gradient purification of the virus. This property has made clone E11S a valuable reagent for the isolation of large quantities of purified gp120 and for examination of the vaccine potential of SIV's with native gp120. Clone E11S has been inoculated into 12 M. nemestrina by various routes, and although the study is not completed, is pathogenic in those macaques.

A full-length molecular clone of SIV/Mne (clone 5) has been obtained by screening a lambda library of infected HuT-78 cells. This molecular clone is infectious in vitro after transfection of HuT-78 cells and has been inoculated intravenously into two macaques and two baboons. The baboons did not become infected, but both macaques seroconverted and at 60 weeks one animal had a marked decrease in $CD4^+$ cells (10% of normal levels), which is frequently an indicator of impending opportunistic infections. This molecular clone has been completely sequenced; clone 5 is 95% related overall to SIV/Mac, (another macaque isolate), 82% to HIV-2 and 41% to HIV-1.

4. Identification of a cell line that produces a marked cytopathic effect after infection with SIV's. Several cell lines readily form syncytia and giant cells after infection with HIV-1. These same cells do not show any cytopathic changes after infection by SIV's, although SIV's often replicate

readily as determined by reverse transcriptase assays. We have now identified an Epstein-Barr virus-transformed human cell line and developed a clone that forms giant cells within 48 hours of infection by SIV/Mne, SIV/Cat, or SIV/Cae. This cell line, in fact, replicates SIV's more readily than HIV-1. We have used this cell line to develop a rapid (5-day) assay to test for neutralizing activity in primate sera from infected animals.

Publications:

Benveniste RE, Morton WR, Clark EA, Tsai C-C, Ochs HD, Ward JM, Kuller L, Knott WB, Hill RW, Gale MJ, Thouless ME. Inoculation of baboons and macaques with simian immunodeficiency virus/Mne, a primate lentivirus closely related to human immunodeficiency virus type 2. J Virol 1988;62:2091-101.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05417-05 LVC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Characterization and Expression of raf Oncogenes in Normal and Tumor Cells

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Others:	Ulrich Brennscheidt	Special Volunteer	LVC	NCI
	Jong-Eun Lee	Special Volunteer	LVC	NCI
	Guna Sithanandam	IRTA Fellow	LVC	NCI
	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
	Stephen Storm	Biologist	LVC	NCI
	Berton Zbar	Chief, Cellular Immunity Section	LI	NCI
	Adi Gazdar	Chief, Human Tumor Cell Biol. Sect.	NMOB	NCI

COOPERATING UNITS *(if any)*

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LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.9	1.8	1.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Three functional proto-oncogenes related to v-raf have been identified in man; these have been designated A-raf, B-raf, and c-raf. c-raf-1 has been localized to human chromosome 3p25 near sites specifically altered in small cell lung carcinoma (SCLC), familial renal cell carcinoma (RCC), and mixed parotid gland tumors. All 17 exons of the human c-raf-1 gene have been cloned and shown to span more than 75 Kbp. c-raf-1 is expressed as a 3.4-Kb RNA which is found in all tissues and cell lines examined, although the levels vary as much as fivefold. Consistent with its ubiquitous expression, the c-raf-1 gene promoter has the features of a house-keeping gene in that it is GC-rich, lacks a TATA-box, and contains several SP1 binding sites. However, an octamer binding motif is located at -500 bp. Using restriction fragment length polymorphisms (RFLPs) located within the c-raf-1 locus, we examined DNA from 84 human lung carcinomas and have determined that one allele of c-raf-1 is deleted in SCLC. Linkage analysis has demonstrated that the c-raf-1 locus is located within 13 cM of the autosomal dominant gene for Von Hippel-Lindau disease, a syndrome which results in an inherited susceptability to certain cancers, including RCC. A-raf-1 has been localized to the X chromosome, p11.4-cen, near a specific translocation site consistently found in synovial carcinoma and also near genetic loci for Wiscott-Aldrich and Norrie syndromes. A-raf-1 is a more compact gene than c-raf-1, which may explain why it has not registered as a DNA-transfection activated oncogene. The A-raf mRNA is 2.6 Kb, it encodes a 606 amino acid phosphoprotein (67.5 Kd) which shows 60% homology with c-raf-1 and displays a more restricted pattern of tissue expression than c-raf-1, with highest levels in the epididymis. The A-raf promoter displays features of a regulated eukaryotic promoter in that it contains a TFIID binding site (TATA-box), sequences identical to the binding sites of several transcription factors found in adenovirus early gene promoters, and a glucocorticoid response element located within exon 1.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Ulrich Brennscheidt	Special Volunteer	LVC	NCI
Jong-Eun Lee	Special Volunteer	LVC	NCI
Guna Sithanandam	IRTA Fellow	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Stephen Storm	Biologist	LVC	NCI
Berton Zbar	Chief, Cellular Immunity Section	LI	NCI
Adi Gazdar	Chief, Human Tumor Cell Biology Section	NMOB	NCI
John Minna	Chief	NMOB	NCI

Objectives:

The specific objectives of these studies are (1) to molecularly characterize raf genes in normal and tumor tissues, (2) to determine how these genes are regulated in normal and transformed cells, (3) to identify and characterize the genes and gene products which regulate raf expression in normal and transformed cells, and (4) to define the genetic mechanism(s) by which the raf family of proto-oncogenes becomes activated and causes transformation.

Methods Employed:

Standard recombinant DNA techniques were used to molecularly clone and sequence raf cDNA species and genomic DNA clones. raf gene polymorphisms were analyzed using a panel of 30 unrelated Caucasians and segregation confirmed in a three-generation pedigree. Protein analyses of raf in normal and transformed cells were carried out by metabolic labeling, immunoprecipitation, and gel analyses; raf-associated kinase activity was demonstrated using our established protocol.

Major Findings:

1. Near full-length cDNAs for the human c-raf-1 gene have been isolated from a human fetal liver and a small cell lung carcinoma (SCLC) cell line. Both cDNAs are approximately 3 Kb in length and share identical restriction patterns. The fetal liver cDNA contains a single large open reading frame (ORF) of 1944 nucleotides coding for a phosphoprotein of 648 amino acids and a predicted molecular weight of 73.0 Kd. Using additional cDNA libraries, we have isolated 5′ extensions relative to the original fetal liver cDNA; these have allowed the isolation of genomic clones containing all 17 exons of the human c-raf-1 gene. These apparently span more than 75 Kb and suggest that the untranslated sequences from exon 1 are displaced by at least 35 Kb from the main body of the gene (exons 2-17) which spans 40 Kb.

2. The c-raf-1 oncogene is located at chromosome 3p25, near a region known to be specifically deleted in patients with renal cell carcinoma and SCLC. From the cytogenetic analysis of SCLC, we have estimated that one allele of the

c-raf-1 locus was deleted in approximately 80% of the cases. However, c-raf-1 was generally thought to be distal to the most common deletion in SCLC, 3p14-23. Using restriction fragment length polymorphisms (RFLPs) located within the c-raf-1 locus, we have examined DNA from 84 human lung carcinomas. In an analysis of 11 paired (normal versus tumor) SCLC DNA samples, all 5 informative cases showed loss of heterozygosity at this locus in the corresponding tumor sample. Analysis of 73 unpaired lung carcinoma DNAs showed that out of 31 non-SCLC samples, 48% were heterozygous for the Bgl I polymorphism and 23% showed heterozygosity with Taq I; however, all of the 42 samples were homozygous for both of these RFLPs. This striking loss of heterozygosity at the c-raf-1 locus in SCLC indicates that one allele of c-raf-1 is deleted in SCLC. To determine if the c-raf-1 gene product is altered in SCLC, we have analyzed RNA and protein from several SCLC cell lines. Using extracts from these cells in immune complex kinase assays, the kinase activity of the c-raf protein appears to be constitutively activated. Whether this apparent activation results from genetic or epigenetic events is under investigation.

3. c-raf-1 gene expression has been examined in adult and embryonic mouse tissues and in established cell lines using a variety of mitogens and growth inhibitors by Northern hybridization. The gene is uniformly expressed in most cells and tissues examined, albeit at levels that vary by fivefold. The mRNA size is 3.4 and 3.1 Kb in human and mouse, respectively. We have only observed altered c-raf mRNAs in one mouse cell line containing the long terminal repeat-activated c-raf-1; however, altered cDNAs which apparently result from differential splicing have been identified which would not be predicted to deviate significantly from the normal size (3.4 Kb) c-raf-1 mRNA. c-raf RNAs are uniformly expressed at very high levels in chemically-induced mouse lung carcinomas and lymphomas, in transformed murine and human tumor cell lines, and in chemically-induced rat liver preneoplastic nodules, but not necessarily in the resulting rat hepatomas.

4. The isolation of genomic clones containing exon 1 sequences of the human c-raf-1 gene has allowed us to map the 5′ end of the c-raf RNA using S1 and primer extension analyses, obtain the nucleotide sequence of the c-raf-1 promoter region, and functionally characterize its promoter activity by linkage to reporter genes. From these studies, we conclude that the c-raf promoter has the structural features of a eukaryotic housekeeping gene in that it contains multiple RNA start sites, lacks a TATA-box, is GC-rich and contains several SP1 binding sites. Preliminary data using the c-raf promoter linked to a CAT gene reporter suggest a weak promoter activity in transient transfection assays.

5. A near full-length human A-raf cDNA was isolated from the T-cell library which is 2.46 Kb and contains a single long ORF of 1818 nucleotides coding for a protein of 606 amino acids and a molecular weight of 67.5 Kd. The predicted amino acid sequence shows 60% identity with the c-raf-1 and, taking into account conservative amino acid substitutions, the homology is greater than 85%. Southern blotting experiments and genomic cloning suggest that the A-raf-1 gene is less than 37 Kb and that very short introns intersperse the coding sequences, at least in the 5′ half of the gene. The smaller size of this gene relative to c-raf-1 suggests a possible reason why A-raf-1 has not been detected as an oncogene in DNA transfection experiments.

6. The A-raf-1 locus has been mapped in both mouse and man to the X chromosome. The human A-raf-1 locus has been regionally localized to p11.4-cen, near a specific translocation t(X;18)(p11.2;q11.2) in human synovial carcinomas and the genes for Wiscott-Aldrich (between to DXS14 and DXS7) and Norrie (linked to DXS7) syndromes. In addition, rare translocations of the X chromosome with autosomes have been observed and X chromosome loss is a frequent occurrence in certain types of acute lymphocytic leukemia. In the mouse, the A-raf-1 locus has been mapped to a region 10-17 cM proximal to the hypoxanthine phosphoribosyl transferase gene between the DXPas4 locus and the DXPas7 locus. This localization is compatible with the presence of the A-raf-1 oncogene on the short arm of the human X chromosome between the centromere and Xp11.4. More precise localization of the human A-raf-1 gene using RFLP analysis is clearly required for assessment of its potential role in cancer.

7. A-raf gene expression has been examined in embryonic and adult mouse tissues, and in a variety of murine and human cell lines. The A-raf mRNA is 2.6 Kb in both rodents and humans. However, in certain human and murine T-cell lines a 1.3-Kb A-raf hybridizing mRNA has also been observed. In contrast to c-raf-1, A-raf-1 shows a restricted tissue distribution of expression and is generally expressed at a lower level than c-raf except in the epididymis where the highest levels of A-raf are detected at levels approximately fivefold greater than c-raf.

8. The isolation of genomic clones encompassing the 5′ half of the human A-raf-1 gene has allowed the characterization of its promoter region. The A-raf promoter, unlike c-raf, displays features of a regulated eukaryotic promoter in that it contains a TFIID binding site (TATA-box), SP1 sites, binding sites for transcription factors, E2aE-C (AATTCCCA) and E4TF1 (CACTTCC), found in the promoter regions of several adenovirus early gene promoter, and a consensus glucocorticoid response element (TGTTCT) is located within exon 1. The A-raf promoter region directs the expression of a beta-globin reporter gene in transient transfection assays.

Publications:

Beer DG, Neveu MJ, Paul DL, Rapp UR, Pitot HC. Expression of the c-raf proto-oncogene and a gap junction protein in preneoplastic foci and rat liver neoplasms. Cancer Res 1988;48:1610-7.

Sithanandam G, Dean M, Brennscheidt U, Beck T, Gazdar A, Minna JD, Brauch H, Zbar B, Rapp UR. Loss of heterozygosity at the c-raf locus small cell lung carcinoma. Oncogene 1989;4:451-5.

Sithanandam G, Heidecker G, Beck T, Minna JD, Zbar B, Rapp UR. Loss of heterozygosity at the c-raf-1 locus and analysis of c-raf protein in small cell lung carcinoma. In: Furth MD, Greaves MF, eds. Cancer cells: molecular diagnostics of human cancer. New York: Cold Spring Harbor Press (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05434-05 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Immunology of AIDS and AIDS-Related Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Dean L. Mann	Chief, Immunogenetics Section	LVC	NCI
Others:	William Blattner	Chief, Family Studies Section	EEB	NCI
	James J. Goedert	Coordinator, AIDS Working Group	EEB	NCI
	Robert J. Bigger	Medical Officer	EEB	NCI
	Mikulas Popovic	Medical Officer	LTCB	NCI

COOPERATING UNITS *(if any)*
Food and Drug Administration, Bethesda, MD (D. Shepp)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Immunogenetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.9	0.6	0.3

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Studies are being conducted to investigate the immunobiology of human immunodeficiency virus (HIV)-1 infection. The primary target cells for infection are the CD4+ T cells and monocyte/macrophage (MM). One of the cells of the MM lineage, the skin Langerhans' cell, was shown to be infected in vivo in skin biopsies. Cells of this type were isolated and demonstrated to be susceptible to infection and propagation of HIV-1 in vitro. MM to T cell transmission of HIV-1 was studied in vitro. MM infected with HIV-1 were more efficient than cell-free virus in infecting autologous T cells. Infected MM also presented exogenous antigens, tetanus toxoid, and streptokinase to autologous T cells comparable to noninfected MM. Infection of T cells could be blocked with antibodies to the major histocompatibility complex (MHC) class II surface structure that presents antigens to the responding T cells. Cell-mediated cytotoxicity studies were performed using two different target cells expressing HIV-1 or viral proteins. Fibroblasts transfected with HIV-1 were killed by lymphocytes from HIV-1-infected individuals and appeared to be restricted by MHC class I antigens. Using the continuous T cell lines, H9 and H9-HIV-1, as targets and lymphocytes from HIV-1-infected individuals as effector cells matched for class I or class II MHC antigens, cell-mediated cytotoxicity was found to be MHC class II restricted and mediated by CD4+ T cells. Cytotoxicity was lost when patients developed acquired immune deficiency syndrome.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Dean L. Mann	Chief, Immunogenetics Section	LHC	NCI
William Blattner	Chief, Family Studies Section	EEB	NCI
James J. Goedert	Coordinator, AIDS Working Group	EEB	NCI
Robert J. Bigger	Medical Officer	EEB	NCI
Mikulas Popovic	Medical Officer	LTCB	NCI

Objectives:

The objectives of these investigations are to study the immunobiology of human immunodeficiency virus (HIV)-1 infection that leads to acquired immune deficiency syndrome (AIDS) and AIDS-related diseases. These studies include investigation of virus infection of various cell types and comparing the function of infected and noninfected cells. In addition, cell to cell transmission of HIV-1 and the mechanism of destruction of infected cells are being investigated. The studies are also directed at understanding the nature of the immune response of individuals who are infected with this retrovirus and the changes that occur with disease progression.

Methods Employed:

Peripheral blood lymphocytes (PBLs) used in these studies were obtained from individuals infected with HIV-1 and from individuals who were HIV-1 seronegative. Skin biopsies were taken from HIV-1-infected individuals and Langerhans' cells were isolated. These cells were cocultured with monocyte/macrophage (MM) from seronegative individuals, the virus rescued in these cells and then, in turn, cocultured with isolated Langerhans' cells from seronegative individuals.

MM isolated from PBLs from seronegative individuals were infected with HIV-1. Viruses produced by these cells and present in the culture media were used to infect T lymphocytes. Infected MM were cocultured with autologous T cells and monitored for infection at various time periods by determining HIV-1 reverse transcription (RT) activity. Infected and noninfected MM were exposed to antigens, tetanus toxin (TT), or streptokinase for 24 hours, the cells washed and exposed to lymphocytes depleted of MM for 24 hours. Monoclonal antibodies to major histocompatibility complex (MHC) class I and II antigens were added to the cocultures during the period of exposure of T cells to virus-infected or noninfected antigen-pulsed or antigen-free MM. Antigen response was measured by ^{3}H thymidine incorporation in T cells and infection by RT activity.

PBLs from HIV-1-infected individuals were cultured for 7 days, removed and washed, and tested for cell-mediated cytotoxicity. The target cells (H9 and H9-HIV-1) were labeled with ^{51}Cr and cultured for 4 hours with the effector cells (cultured PBLs) at effector target (ET) ratios of 1:100, 1:50, and 1:25. ^{51}Cr release was measured to indicate cytotoxicity. PBLs were depleted of

$CD4^+$ cells by immune selection on antibody-coated beads, and the remaining cells tested for cytotoxic activity to the target cell population.

Major Findings:

Using immunohistochemical techniques, HIV-1-infected Langerhans' cells were readily identified on the skin biopsies from patients with AIDS, or at risk for AIDS. HIV-1 infection was demonstrated with antibodies to gag proteins and the presence of intracellular and budding virus particles. HIV-1 was transferred from these primary biopsies to monocytes in culture. These monocytes in turn were able to infect Langerhans' cells isolated from skin of HIV-1 seronegative individuals. These studies indicate that Langerhans' cells can be infected with HIV-1 and thus may serve as a reservoir for HIV.

Cell to cell (MM to T cell) transmission of HIV-1 was found to be more efficient than cell-free virus infection of T cells. HIV-1-infected MM were capable of presenting TT and streptokinase to autologous T cells comparable to noninfected MM. The T cell infection observed was greater when the antigen (TT) was presented by HIV-1-infected MM compared to that found when HIV-1-infected MM were cultured with T cells without exogenous antigen. Antigen presentation and infection of T cells was blocked by monoclonal antibodies to MHC class II molecules, Dextran SO_4, and the HIV-1 large envelope glycoprotein, gp120.

In the cell-mediated cytotoxicity (CMC) studies, the PBLs to be tested were selected on the basis of the HLA phenotype that was shared with the target cells, H9 and H9-HIV-1. Specific CMC was found only when MHC class II antigens, HLA-DR4, DQW3, and DR53, matched with the targets. No specific CMC was observed with MHC class II identical PBLs from HIV-1 seronegative male homosexuals. Removal of the $CD4^+$ cells was associated with the loss of specific cytotoxicity. In addition, lymphocytes from individuals that demonstrated cytotoxicity early in their disease course lost this cytotoxicity after developing AIDS.

Publications:

Mann DL, Read-Connole E, Arthur LO, Robey WG, Wernet P, Schneider EM, Blattner WA, Popovic M. HLA-DR is involved in the human immunodeficiency virus binding site on cells expressing MHC class II antigens. J Immunol 1988;141:1131-7.

Rappersberger K, Gartner S, Schenk P, Stingl G, Groh V, Tschachler E, Mann DL, Wolff K, Konrad K, Popovic M. Langerhans' cells are an actual site of HIV-1 replication. Intervirology 1988;29:185-94.

Shepp DH, Daguillard F, Mann D, Quinnan GV. Human class I MHC restricted cytotoxic T lymphocytes specific for human immunodeficiency virus envelope antigens. AIDS 1988;2:115-7.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05528-03 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 charecters or less Title must fit on one line between the borders.)*
Mechanisms of the HTLV-I and BLV rex Proteins

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: David Derse Senior Staff Fellow LVC NCI

Others: None

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (L. Martarano)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS.	PROFESSIONAL.	OTHER.
0.4	0.3	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Human T lymphotropic virus-I (HTLV-I) and bovine leukemia virus (BLV) are closely related retroviruses that use similar strategies to regulate gene expression. They both encode two regulatory proteins, termed tax and rex, that interact with specific cis-acting sequences to control transcription initiation and RNA accumulation, respectively. This laboratory previously showed that the BLV rex protein was required for the synthesis of viral mRNAs and that sequence elements in the 3′ long terminal repeat (LTR) mediated this effect. To better understand the molecular basis of rex action, HTLV-I and BLV rex proteins (which are approximately 40% identical) were examined in parallel. HTLV-I was molecularly cloned from a cell line originating from a patient with adult T-cell leukemia. Plasmids were then constructed to test rex functions in transfected cells. HTLV-I rex, like BLV rex, was required for synthesis of viral RNAs and interacts with elements in the 3′ LTR. The BLV rex protein was found to complement rex-deficient HTLV-I provirus gene expression and vice versa. The LTRs of both viruses were responsive to the rex protein of the other virus, although activity was greater with the homologous protein. Northern blot analysis suggests that rex probably acts in RNA processing rather than transport. Rabbit antisera were raised against synthetic peptides representing either HTLV-I or BLV rex. Immunoprecipitations revealed that multiple rex species are present in cells transfected with rex-expression plasmids, suggesting that multiple initiation codons and possibly post-translational modifications contribute to rex heterogeneity. Rex proteins are now being examined in cell-free systems to establish molecular mechanisms.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

David Derse Senior Staff Fellow LVC NCI

Objectives:

1. Construct a molecular clone of human T lymphotropic virus-I (HTLV-I) from which to generate provirus mutants deficient in regulatory gene expression, plasmids that express the regulatory proteins tax and rex, and other subgenomic expression and test plasmids.

2. Compare the activities and specificities of the HTLV-I and bovine leukemia virus (BLV) rex proteins on the various rex-response elements. Test the activities of HTLV-I/BLV chimeric rex proteins.

3. Characterize the BLV and HTLV-I rex proteins expressed in transfected cells by radioimmunoprecipitation analysis.

4. Establish cell-free systems to examine rex function and mechanism.

Methods Employed:

The following methods were employed: (1) cloning in bacteriophage lambda, (2) nucleotide sequencing, (3) recombinant plasmid construction, (4) transfection of mammalian cells, (5) Northern blotting, (6) radioimmunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and (7) in vitro transcription and translation of virus genes.

Major Findings:

1. Cross-reactivity of HTLV-I and BLV rex proteins. Two approaches have been developed to monitor rex activity in transfected mammalian cells. In the first method, RNA synthesis directed by a provirus mutant, deficient in rex production, is complemented using a rex-expression plasmid. It was previously observed with BLV, and has now been shown for HTLV-I, that rex is required in trans for the accumulation of mRNAs encoding the structural proteins, gag, pol and env. Furthermore, it was found that HTLV-I rex would complement a rex$^-$ BLV mutant and that BLV rex would complement a rex$^-$ HTLV-I mutant. In the second approach, either the BLV or HTLV-I long terminal repeat (LTR) was placed 3′ of a rabbit β-globin cDNA controlled by the Rous sarcoma virus promoter; accumulation of β-globin RNA in transfected cells was greatly increased in response to rex expression. The BLV LTR was responsive to BLV and HTLV-I rex and the HTLV-I LTR was responsive to both HTLV-I and BLV rex proteins; in both cases response was greater using the homologous rex protein. This cross-activation is interesting in view of the fact that the rex proteins are only 30% to 40% similar and that the LTRs, containing the rex-response elements, are even more divergent. The RNAs transcribed from the 3′ LTRs may, however, adopt similar secondary structures. These cis-acting elements in the

LTRs are currently being altered by mutagenesis to examine the structure-function relationships.

Chimeric rex proteins have been constructed in which the amino-terminal 20 residues have been exchanged between BLV and HTLV-I; this region is the most highly conserved between the two proteins. The activities of these reciprocal recombinants were compared in both systems described above. It appears that the highest activity was observed on the BLV LTR using the rex protein composed of the HTLV-I amino terminus on the BLV body; the converse was true using the HTLV-I LTR. These results suggested that the active site of rex is located downstream of the amino-terminal 20 amino acids.

An interesting offshoot of these experiments has been the observation that HTLV-I but not BLV rex will complement an HIV-1 mutant defective in rev production. HIV-1 is very distantly related to the HTLV-I/BLV group of viruses and encodes several regulatory proteins; the rev and rex proteins do not share any obvious regions of sequence similarity. The converse complementation, i.e., HIV-1 rev on a rex$^-$ HTLV-I mutant, was not observed. The basis for this phenomenon is currently under study.

2. Characterization of HTLV-I and BLV rex proteins expressed in transfected mammalian cells, bacteria and cell-free systems. Rabbit antisera has been raised against synthetic peptides deduced from the sequences of BLV and HTLV-I rex genes. These are being used to immunoprecipitate labelled proteins produced in in vitro translation systems, bacteria and transfected mammalian cells. The BLV and HTLV-I rex genes were inserted into plasmids adjacent to bacteriophage RNA polymerase promoters so that rex RNA could be synthesized in vitro. These RNAs were used to charge rabbit reticulocyte lysate translation reactions in the presence of (S-35)methionine. Labelled proteins were immunoprecipitated with antibodies to rex and fractionated by denaturing gel electrophoresis. These experiments revealed that the rabbit antisera, raised against synthetic peptides, recognized the rex proteins. Both BLV and HTLV-I rex RNAs, translated in vitro, yielded two proteins corresponding to initiation at the 5′ terminus and at an AUG codon within the rex gene. For BLV, the proteins run at 17,000 MW and 13,000 MW; for HTLV-I the apparent sizes are approximately 21,000 MW and 16,000 MW. This cell-free translation system has also been useful in assessing the translation efficiencies of the various initiation codons in these tricistronic RNAs. For example, the HTLV-I rex initiation codon is inefficient compared to BLV and can be greatly improved by altering nucleotides near this AUG; increasing the efficiency of this 5′ terminal initiator decreases the yield of the smaller protein which is initiated downstream, however. The rex proteins have also been produced in a bacterial expression system in which the rex genes are fused to a bacteriophage T7 promoter and made in great excess in response to infection with phage that supplies T7 RNA polymerase. Both the bacterially expressed and the in vitro synthesized rex proteins will be used in in vitro systems to examine rex protein modification and activity.

Immunoprecipitation of rex proteins from (S-35)methionine-labelled mammalian cells has revealed that rex is posttranslationally modified. In cells transfected with BLV rex-expression plasmids, a protein of about 20,000 MW was observed in addition to the two proteins described above. Similar results are

seen with HTLV-I; i.e., an additional protein of larger size is precipitated. The types of modifications and the intracellular locations of the various species are currently being examined.

3. In vitro systems designed to examine molecular mechanisms of rex. As discussed above, it is still unclear how rex interacts with cis-acting elements in the 3′ LTR and how regulation is effected. Experiments have just begun in which rex proteins, produced in cell-free translation systems or in bacteria, are combined with labelled RNA synthesized in vitro. The possible association of rex with specific RNAs is monitored by gel retardation or by UV crosslinking. Similar approaches are currently being used to examine rex activity in nuclear extracts of mammalian cells expressing rex.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05529-03 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetic and Molecular Organization of the MHC in the Domestic Cat

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Naoya Yuhki	Visiting Associate	LVC	NCI
Others:	Stephen J. O'Brien	Chief	LVC	NCI
	Janice S. Martenson	Microbiologist	LVC	NCI
	Mary E. Eichelberger	Microbiologist	LVC	NCI
	Stanley J. Cevario	Biologist	LVC	NCI

COOPERATING UNITS *(if any)*
Program Resources Inc., Frederick, MD (C. A. Winkler)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.6	0.6	1.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The feline major histocompatibility complex (MHC) has been studied using serological and molecular techniques as an approach to comparative genome organization of this important gene cluster. The MHC in most mammals consists of two classes of genes, classes I and II, which play special roles in presenting antigens to T-cell receptors. Skin graft experiments revealed serological polymorphism for both class I and class II alleles as defined by immunoprecipitation. Cluster analysis of 13 alloantisera using outbred cats revealed six associated clusters representing group antigen specificities. Cell hybrid analysis permitted gene mapping of the feline MHC, termed FLA, to chromosome B2. Genomic and cDNA clones of class I FLA genes were derived and sequence analysis revealed striking homology with human and murine MHC class I sequences. Sequence analysis of seven different FLA class I cDNA transcripts revealed some interesting differences between cat FLA and other species; e.g., in the domestic cat: (1) a cysteine residue of the cytoplasmic domain was lacking, (2) a cluster of amino acid substitutions was observed in a highly constant region of the α2 domain, and (3) no amino acid substitutions were observed in a highly variable region of the α2 domain. Comparison of the seven cDNA transcripts suggest that intragenic recombination has played a major role in generation of MHC diversity in the cat. Two different types of DNA recombinations act to create a modern polymorphism of feline MHC class I genes. These DNA recombinations include at least one unequal (non) crossing over at the points in the center of the first α-helix coding region in the α1 domain and perhaps one between the exons encoding α1 and α2 extra-cellular domains. The highly conserved 23 b.p. sequence laid on the recombination point in the first α-helix coding region which suggests this sequence is an intraexon recombination hot spot.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Naoya Yuhki	Visiting Associate	LVC	NCI
Stephen J. O'Brien	Chief	LVC	NCI
Janice S. Martenson	Microbiologist	LVC	NCI
Mary E. Eichelberger	Microbiologist	LVC	NCI
Stanley J. Cevario	Biologist	LVC	NCI

Objectives:

The major histocompatibility complex (MHC) genes encode two different classes of cell surface molecules which can present immunological peptides for T-cell receptors (class I and class II antigens). These molecules have highly polymorphic features. According to the x-ray crystallographic model of a human HLA-A2 class I molecule, the majority of polymorphic residues of this molecule are located on the site facing the putative antigen binding site in human and mouse class I molecules. This finding, together with the results that various HLA class I molecules have different binding capacities than immunological peptides, suggests that the polymorphism of MHC class I molecules reflects the capacity of each molecule to bind various spectrums of immunological peptides. Several theories, such as gene conversion, in vivo exon shuffling and overdominant selection have been proposed to explain the extreme polymorphism of MHC molecules by analyzing human and mouse MHC genes or gene products. We used a different animal model, the domestic cat, to examine the mechanisms.

Methods Employed:

The following techniques were employed: 1) cDNA cloning, 2) DNA sequencing using the dideoxy nucleotide sequencing method, and 3) RNA and DNA blotting.

Major Findings:

1. Serological characterization of FLA, the feline MHC. The MHC of the domestic cat (termed FLA) has been refractile to genetic and serological definition largely because of repeated failure to detect cytotoxic antibodies in multiparous cats or to elicit antibody following allogeneic lymphocyte immunization. We have developed a protocol for producing cytotoxic alloantisera in the cat following rejection of multiple surgical skin grafts. Of 59 cats subjected to grafting, 13 produced lymphocytotoxic antisera which had varying specificities among a panel of outbred cat cells. A population cluster analysis of the 13 alloantisera permitted the identification of six clusters of overlapping FLA specificities. Serological analysis of cells from 12 cat kindreds led to the definition of 24 allogeneic haplotypes, which segregate as a single Mendelian complex. Feline anti-FLA sera were characterized as class I- or class II-specific by immunoprecipitation of FLA gene products on lymphocyte cell surfaces. Abundant antigenic polymorphisms for both class I and class II MHC determinants were discovered, a result

consistent with precedence in other species and the common expectation of the adaptive value of MHC variation. Development of feline MHC typing reagents and the definition of haplotypes for the cat hold promise for experimental analysis of valuable feline models for virus-induced immune deficiencies.

2. Molecular genetic characterization of class I and class II genes of the domestic cat. A comparative analysis of class I and class II genes in domestic cat populations was undertaken using molecular probes of the MHC from man and mouse. The cat possesses a minimum of 20 class I loci and 5 class II genes per haploid genome. Class I genes of the domestic cat expressed limited restriction fragment length polymorphism (RFLP). The average percent difference of the size of DNA fragments between individual cats was 9.0%, a value five times lower than the value for mice, but comparable to the human DNA polymorphism level. Class I and class II genes were both genetically mapped to feline chromosome B2 using a panel of rodent x cat somatic cell hybrids. Since feline chromosome B2 is syntenically homologous to human chromosome 6 and mouse chromosome 17, these results affirm the linkage conservation of the MHC-containing linkage group in the three mammalian orders.

3. Sequence organization of cDNA clones of the feline MHC. The abundant functional polymorphism and evolutionary divergence of mammalian MHC class I genes has been recently affirmed by sequence analysis of over 40 mouse H-2 and human HLA transcripts. In a comparative approach to evolution of the MHC, we isolated eight molecular clones of feline MHC (termed FLA for feline leukocyte antigen) class I genes from a cDNA library of a cat T-cell lymphoma line. DNA sequence analysis of eight clones revealed they all fell into one of two internally identical allelic groups which differed by 9% of their nucleotide sequences. The occurrence of only two allelic cDNA clones is consistent with the expression of a single heterozygous functional class I gene in the studied cell line despite the occurrence of over 20 class I copies estimated to be present in the cat genome. Comparison of the FLA class I coding sequence with other class I genes from other species revealed that the domestic cat genes display 81 to 82% sequence identity with human, and 73 to 79% sequence identity with mouse class I genes. Feline and human class I genes have similar sequences and protein structures with three (α) extracellular domains, one transmembrane domain, and one cytoplasmic domain. Variable codons detected in FLA class I alleles were, in most cases, in positions which were also variable in man and mouse, while invariant positions with defined functional constraints were generally conserved and invariant between the three species as well. Southern analysis of DNA from diverse species of Felidae revealed a similar numerity and restriction pattern indicating conservation of the organization of class I genes during the Felidae radiation.

4. DNA variation of the mammalian MHC reflects genomic diversity, functional diversity of the MHC, general genomic diversity and population natural history. The MHC is a multigene complex of tightly linked homologous genes which encode cell surface antigens that play a key role in immune regulation and response to foreign antigens. In most species, MHC gene products display extreme antigenic polymorphism and their variability has been interpreted to reflect an adaptive strategy for accommodating rapidly evolving infectious

agents which periodically afflict natural populations. Determination of the extent of MHC variation has been limited to populations in which skin grafting is feasible or for which serological reagents have been developed. We present here a quantitative analysis of RFLP of MHC class I genes in several mammalian species (cats, rodents, humans) previously known to have very different levels of genetic diversity based on functional MHC assays and on allozyme surveys. When homologous class I probes were employed, the study demonstrates a notable concordance between the extent of MHC-RFLP variation and functional MHC variation detected by skin grafts or genome-wide diversity estimated by allozyme screens. These results confirm the genetically depauperate character of the African cheetah, Acinonyx jubatus, and the Asiatic lion, Panthera leo persica; further, they support the use of class I MHC molecular reagents in estimating the extent and character of genetic diversity in natural populations.

5. Two different types of DNA recombination events are involved in the creation of polymorphism of feline class I molecules. The traces of two different types of DNA recombination events were found in the sequences of feline class I clones. One apparent DNA recombination occurred in the center of the first α-helix coding region. Six clones had mosaic sequences of one donor clone and an unidentified gene in this region. Highly conserved 23 b.p. sequences were located between the recombinant sequences. This evidence suggests that this conserved sequence acts as a recombination hot spot of the class I molecules.

6. Overdominant selection governs the polymorphism of feline class I molecules. Recently it has been proposed that overdominant selection is an important factor for the creation of polymorphism of MHC class I molecules in human and mouse. This conclusion is based on the observation that the rate of nucleotide substitution in the antigen binding site is much greater than in other domains of the class I molecules. The rates of non-synonymous (codon altering) substitution were significantly higher than synonymous (non-codon altering) substitution in the antigen binding site; but the reverse is true in the other region in human and mouse class I molecules. These observations were interpreted to indicate that polymorphic residues in the antigen binding site are positively selected during evolution because of the advantages for binding a certain spectrum of immunological peptides. Using feline class I molecules, we confirmed and extended that theory by comparative analysis of multiple transcripts. From these results, we speculate that MHC class I molecules were evolved by the combinations of simple mutations, DNA recombinations, and overdominant selection.

Publications:

Winkler C, Schultz A, Cevario S, O'Brien S. Genetic characterization of FLA, the cat major histocompatibility complex. Proc Natl Acad Sci USA 1989;86: 943-7.

Winkler C, Yuhki N, O'Brien SJ. The major histocompatibility of the felidae. In: Clegg M, O'Brien SJ, eds. Proceedings of the UCLA symposium on molecular and cellular biology. New York, Alan R Liss (In Press).

Yuhki N, Heidecker GF, O'Brien SJ. Characterization of MHC cDNA clones in the domestic cat: diversity and evolution of class I genes. J Immunol 1989;142: 3676-82.

Yuhki N, O'Brien SJ. DNA variation of the mammalian MHC reflects genomic diversity and population history. Proc Natl Acad Sci USA (In Press).

Yuhki N, O'Brien SJ. Molecular characterization and genetic mapping of class I and class II MHC genes of the domestic cat. Immunogenetics 1988;27:414-25.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05531-03 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Functional Characterization of the Relationship Between raf and Protein Kinase C

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Others:	Walter Kolch	Visiting Fellow	LVC	NCI
	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

COOPERATING UNITS (if any)
Program Resources, Inc., Frederick, MD (P. Lloyd, S.D. Showalter); Genetics Institute, Cambridge, MA (J. Knopf)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.8	0.7	0.1

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Cytoplasmic serine/threonine-specific kinases, such as raf and protein kinase C (PKC), seem to play an integrative role in the processing of mitogenic stimuli that originate from membrane-bound growth factor signal transduction systems. To study the role of these kinases in growth regulation, recombinant retroviruses were constructed, which express either a full-length PKC or a gag-PKC fusion protein that possesses constitutive kinase activity. These viruses did not transform NIH 3T3 cells; however, they caused a change in the pattern of raf-associated phosphoproteins. Chimeric raf/PKC viruses are currently being tested for their effects on NIH 3T3 cells. These constructs are expected to be very useful for the characterization of substrates and ligands for either kinase. To specifically inhibit raf function, vectors expressing raf antisense RNA were made. While no effect on raf mRNA levels were observed, levels of c-raf protein were reduced in clones expressing high levels of antisense RNA. Preliminary growth kinetic experiments indicate a negative effect of raf antisense RNA on cell growth. In an alternative approach, we produced monoclonal antibodies against the raf kinase domain, which are currently being tested in microinjection assays for their ability to inhibit raf function.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Walter Kolch	Visiting Fellow	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

Objectives:

The goals of this study are to investigate the role and mutual interaction of the serine/threonine-specific protein kinases, raf and protein kinase C (PKC), in growth factor signal transduction. The experimental strategy is based on (1) the construction of PKC and PKC + raf retroviruses to test for raf - PKC synergism/antagonism, (2) the construction of raf/PKC chimeric viruses to compare domain function and substrate/ligand binding, and (3) specific inhibition of raf function to be able to dissect functional equivalence and divergence between raf and PKC.

Methods Employed:

Standard recombinant DNA cloning methods were used. The expression of transfected virus constructs is monitored by Northern and Western blotting. Production of monoclonal antibodies followed standard procedures.

Major Findings:

Several lines of evidence indicate that the similar structural organization shared by raf family kinases and the PKC family may be reflected in a functional relationship. For instance, 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment of fibroblasts causes hyperphosphorylation and activation of the c-raf kinase. Even more striking, the raf-mediated T-cell response triggered by stimulation of Thy-1 with monoclonal antibodies is dependent on PKC. To explore and compare the function of corresponding domains in raf and PKC, a series of 3611-murine sarcoma virus (MSV)-based, neomycin-selectable retroviruses is being made. SW-1 expresses a full-length PKCalpha cDNA; PKCneo61 expresses a gag-PKC fusion protein analogous to the gag-raf fusion protein expressed by 3611-MSV. Neither PKC virus transformed NIH 3T3 cells, although PKCneo61 exhibits constitutive kinase activity independent of TPA and Ca^{++}. However, analysis of raf- and PKC-associated phosphoproteins reveals a higher level of protein phosphorylation in SW-1 and PKCneo61 cells as compared to NIH 3T3 cells as well as a 180-kDa raf-associated protein, which dissociates upon TPA stimulation. These viruses are now being tested for their ability to alter the growth factor requirements of cells. By exchanging corresponding domains, chimeric raf/PKC viruses are constructed. These chimeric kinases are expected to be useful tools for identifying and characterizing substrates and ligands for either raf or PKC. A virus expressing raf plus PKC was made by incorporating an activated version of raf into PKCneo61.

In order to distinguish between PKC effects mediated by raf and raf-independent PKC effects, we are designing reagents capable of specifically impairing raf function. One strategy employed retrovirus vectors expressing raf antisense RNA. Computer modeling of the folded structure of the c-raf mRNA was used to search for thermodynamically unstable regions, which are likely to serve as suitable targets for antisense RNA. Predicted oligonucleotides were cloned into pMNC (provided by Dr. Brian Seed), a Moloney-based retrovirus vector with a neomycin resistance gene and an internal cytomegalovirus promoter, which is highly active in a wide variety of mammalian cells. Neomycin-resistant colonies were analyzed for expression of antisense RNA, c-raf mRNA, and c-raf protein. While no effect on raf mRNA was observed, the levels of raf protein inversely correlated with the amount of antisense RNA expressed. Preliminary growth kinetic experiments indicate a negative effect of raf antisense RNA on proliferation. We are now working on optimizing expression of the antisense RNA. In order to use these vectors in neomycin-resistant cells which harbor recombinant raf mutants, we replaced the neomycin resistance gene of pMNC with a puromycin resistance gene as selectable marker. These constructs are currently being tested.

As an alternative strategy we produced monoclonal antibodies against the raf-kinase domain. A truncated v-raf protein expressed in E. coli served as antigen. ELISA-positive clones were further assayed for their ability to react with various forms of raf proteins on Western blots and in immunoprecipitation. Four antibodies recognize exclusively c-raf and v-raf, and one antibody reacts with A-raf, c-raf, and v-raf. Deletion analysis of the expression vector showed that the epitopes of all antibodies map into a 10-kDa peptide. This fragment includes the sequence, APE, which has been shown to be crucial for the function of oncogene protein kinases. Antibodies are now being tested in microinjection assays for their ability to block raf-mediated mitogenesis. Using a full-length c-raf protein as antigen we aim at isolating monoclonal antibodies which will inhibit the mitogen activation of the full-length c-raf protein.

Publications:

Heidecker G, Cleveland JL, Beck T, Huleihel M, Kolch W, Storm SM, Rapp UR. Role of raf and myc oncogenes in signal transduction. In: Colburn N, ed. Genes and signal transduction in multistage carcinogenesis. New York: Marcel Dekker, 1989;339-74.

Kolch W, Bonner TI, Rapp UR. Expression of human c-raf-1 oncogene proteins in E. coli. Biochem Biophys Res Commun 1988;152:1045-9.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05532-03 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Effect of raf Family Protein Kinases on Cell Physiology

PRINCIPAL INVESTIGATOR *(List other professional personnal below the Principal Investigator) (Name, title, laboratory, and institute affiliation)*

PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Others:	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
	Walter Kolch	Visiting Fellow	LVC	NCI
	Stephen Storm	Biologist	LVC	NCI
	Robert Nalewaik	Microbiologist	LVC	NCI
	Robert Bassin	Senior Investigator	LTIB	NCI
	Wayne Anderson	Senior Investigator	LCO	NCI

COOPERATING UNITS *(if any)* PRI, Frederick, MD (T. Beck); Dana Farber Cancer Inst., Boston, MA (T. Roberts); Univ. of California, Howard Hughes Medical Inst., Stanford, CA (D. Morrison, R. Williams); NIH, NICHHD, Bethesda, MD (R. Klausner, J. Siegel); St. Judes Children's Hospital, Memphis, TN (J. Cleveland)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
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INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.5	0.7	0.8

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided)*

There are three active raf protein kinase genes in mammals: c-raf, A-raf, and B-raf. c-raf RNA is expressed in all tissues, although steady-state levels vary fivefold between tissues. B-raf shows the most restricted expression with highest levels in brain and testes, where alternate size RNA's are detected. A-raf and B-raf are independently regulated in a tissue-specific manner. They are generally expressed at lower levels than c-raf, except in epididymis where A-raf RNA levels are five to tenfold greater than c-raf.

We have examined the function of c-raf serine/threonine-specific protein kinase in fibroblastic and lymphoid cells. Structure-function analysis suggests a protein structure model for the unstimulated enzyme in which the active site is buried within the protein. Reversible activation in fibroblastic and lymphoid cells can be achieved by many growth factors or intracellular mitogens which use at least two independent pathways--one involving protein kinase C and another which depends on direct tyrosine phosphorylation of c-raf by transmembrane or intracellular tyrosine kinases. In the case of the platelet-derived growth factor receptor, c-raf protein kinase was shown to directly bind to the activated wild type but not an inactive mutant-receptor. Receptor-mediated activation of c-raf protein kinase is followed by translocation of the normally cytosolic enzyme to the perinuclear area and the nucleus.

Expression of activated raf stimulates the activity of PEA1, a member of the c-jun gene family. Both v-raf and activated forms of either c-raf-1 or A-raf stimulate PEA1, suggesting that phosphorylation is involved in this activation. We conclude that c-raf acts as a shuttle enzyme which connects mitogen-initiated events at the plasma membrane to events in the nucleus, presumably by activity-modulation of transcription factors via phosphorylation.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Walter Kolch	Visiting Fellow	LVC	NCI
Stephen Storm	Biologist	LVC	NCI
Robert Nalewaik	Microbiologist	LVC	NCI
Robert Bassin	Senior Investigator	LTIB	NCI
Wayne Anderson	Senior Investigator	LCO	NCI

Objectives:

The objectives of these studies are (1) to characterize the raf family proteins with respect to their positions in signal transduction and to identify the pathway(s) in which they operate, (2) to determine how these proteins are regulated in normal and transformed cells (by phosphorylation and protein associations), (3) to identify putative ligands which may control raf protein kinase activity, (4) to determine the specific cellular targets of "activated" and normal raf, and (5) to identify raf-regulated genes.

Methods Employed:

Standard recombinant DNA techniques were used to molecularly clone and subclone raf cDNA species into Moloney murine leukemia virus (strain Leuk). Specific cDNA clones were inserted into expression vectors and high levels of synthesis of raf polypeptides in E. coli demonstrated by protein gel and immunoblot analyses. Protein analyses of raf in normal and transformed cells were carried out by metabolic labeling, immunoprecipitation, and gel analyses, and raf-associated kinase activity was demonstrated using our established protocol.

Major Findings:

1. The tissue expression patterns suggest that c-raf functions in a common signal transduction pathway(s), whereas A-raf functions in a pathway(s) normally restricted to a limited number of cell types.

2. A functional assay has been developed in which NIH 3T3 cells become growth arrested (as measured by ^{3}H-thymidine incorporation) by microinjection of ras antibody. This assay can be used to provide insight into signal transduction pathways utilized by the oncogenes. Cells infected with oncogene-containing viruses can be microinjected with ras antibody and assayed for DNA synthesis as a measure of the virus' ability to overcome the arrested growth due to the ras antibody block. In this assay, A-raf-murine sarcoma virus (MSV) and 3611-MSV overcome the antibody block, whereas other oncogenes (e.g., v-sis, v-fms, and v-src) are unable to overcome the antibody block. Another functional assay utilizes flat revertants from Kirsten sarcoma virus-transformed cells (v-ras transformed). These cells were found to be resistant to transformation

by v-ras-containing viruses (Kirsten, Harvey and Balb MSV) and some viruses containing the oncogenes, v-fes and v-src. However, we have found that these cells are susceptible to transformation by A-raf-MSV and 3611-MSV. These results suggest that raf family oncogenes act independent of ras either through a signal transduction pathway not involving ras or one in which raf has a position downstream of ras. To further test this preliminary pathway map we have now isolated raf revertant cell lines.

3. Growth factors and oncogenes induce the phosphorylation and the serine/threonine-specific kinase activity of the proto-oncogene product, c-raf, in mouse 3T3 cells. Expression of the membrane-bound oncogene products encoded by v-fms, v-src, v-sis, polyoma virus middle T antigen, and Ha-ras increased the apparent molecular weight and phosphorylation state of the c-raf protein, while expression of the nuclear oncogene and proto-oncogene products encoded by v-fos and c-myc did not. Changes in electrophoretic mobility and phosphorylation occurred rapidly in response to treatment of cells with platelet-derived growth factor (PDGF), acidic fibroblast growth factor, epidermal growth factor, and 12-O-tetradecanoyl-phorbol-13-acetate, but not insulin. The phosphorylation of the c-raf protein occurred primarily on serine and threonine residues. However, a subpopulation of c-raf molecules was phosphorylated on tyrosine residues in cells transformed by v-src or stimulated with PDGF. Transformation by v-src, or treatment with PDGF or phorbol 12-myristate 13-acetate, activated the c-raf-associated serine/threonine kinase activity as measured in immune-complex kinase assays. Our results suggest that the c-raf protein can be activated by tyrosine and/or serine and threonine phosphorylation as a result of direct or indirect action of membrane-bound oncogene products and growth factor receptors. c-raf activation may thus serve to transduce signals from the membrane to the cytoplasm and perhaps on to the nucleus.

4. We have examined the interaction between the serine/threonine kinase, Raf-1, and the tyrosine kinase PDGF B-receptor. Raf-1 tyrosine phosphorylation and kinase activity were increased by PDGF treatment of 3T3 cells or Chinese hamster ovary cells expressing wild-type PDGF B-receptors, but not mutant receptors defective in transmitting mitogenic signals, suggesting that the increase in Raf-1 kinase activity is a significant event in PDGF-induced mitogenesis. Concurrent with these increases, Raf-1 associated with the ligand-activated PDGF B-receptor. Furthermore, both mammalian Raf-1 and Raf-1 expressed using a recombinant baculoviral vector associated in vitro with baculoviral-expressed receptor. This association was markedly decreased by prior phosphatase treatment of the receptor. Following incubation of partially purified baculoviral-expressed PDGF B-receptor with partially purified Raf-1, Raf-1 became phosphorylated on tyrosine and its serine/threonine kinase activity increased four to sixfold. This is the first demonstration of the direct modulation of a protein activity by a growth factor receptor tyrosine kinase.

Publications:

Cleveland JL, Morse HC III, Rapp UR. myc oncogenes and tumor induction. ISI Atlas Biochem 1988;1:93-100.

Heidecker G, Cleveland JL, Beck T, Huleihel M, Kolch W, Storm SM, Rapp UR. Role of raf and myc oncogenes in signal transduction. In: Colburn N, ed. Mechanisms of signal transduction. New York: Marcel Dekker, 1989;339-74.

Horton WR, Cleveland JL, Rapp UR, Kohno K, Yamada Y, Moiyashita T, Hassell JR. The regulation of genes encoding extracellular matrix proteins: in vitro studies and relationships to teratogenic mechanisms. In: Kimmel GL, Kochar EM, eds. In vitro techniques in developmental toxicology: use in defining mechanism and risk parameter. Boca Ratan: CRC Press (In Press).

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Morse HC III, Rapp UR. Tumorigenic activity of artificially activated c-onc genes. In: Klein G, ed. Cellular oncogene activation. New York: Marcel Dekker, 1988;335-64.

Rapp UR, Heidecker G, Huleihel M, Cleveland JL, Choi WC, Pawson T, Hile JN, Anderson WB. Raf family serine/threonine protein kinases in mitogen signal transduction. Proc Cold Spring Harbor Symp Quant Biol 1988;53:173-84.

Rapp UR, Storm SM, Cleveland JL. Oncogenes and interferon. In: Olsson L, ed. Cancer reviews. Copenhagen: Munksgaard, 1987;34-52.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05533-03 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*
Domains Involved in Regulation of Raf Activity

PRINCIPAL INVESTIGATOR *(List other professional personnal below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Others:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
	Walter Kolch	Visiting Fellow	LVC	NCI
	Robert Nalewaik	Microbiologist	LVC	NCI
	Wayne Anderson	Chief	LCO	NCI

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (F.-M. Duh, P. Lloyd)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL:	OTHER
1.5	0.9	0.6

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The protein products of the raf gene family are cytoplasmic serine/threonine protein kinases. They are active in the transmission of mitogenic signals from membrane-associated tyrosine kinase growth factor receptors to the nucleus. The Raf protein itself is phosphorylated and its kinase is activated by several of the tyrosine kinases. Immunofluorescence studies showed that, upon stimulation, c-Raf protein translocates from a diffuse cytoplasmic distribution to the perinuclear space, and probably to the nucleus. Activation of Raf kinase in turn results in increased transcription from a polyoma enhancer region 1 (PEA1) responsive promoter as demonstrated in co-transfection studies using PEA1 responsive promoter plasmids and raf expression constructs. In the same study we showed that Raf inactivated by mutation did not result in enhanced transcription from PEA1-dependent promoters. Other raf mutants analyzed in the same system showed that their ability to influence PEA1-dependent transcription correlated well with their transforming potential, e.g., a linker-insertion mutant in the serine/threonine-rich conserved region 2, which had a slightly activated transforming potential, was able to elevate the level of PEA1-dependent transcription, although not to the same degree as the oncogenically fully activated v-Raf. To identify which other components of the signal transduction pathway directly interact with Raf, either as activators or as substrates, we are currently transferring our wild-type and mutant raf gene constructs into the baculovirus expression system. Recombinant proteins produced in this system show normal modification and are thus more reliable to interact normally with other cellular components than proteins produced in bacteria.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI
Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Walter Kolch	Visiting Fellow	LVC	NCI
Robert Nalewaik	Microbiologist	LVC	NCI
Wayne Anderson	Chief	LCO	NCI

Objectives:

Raf kinase functions as a link in the signal transduction chain relaying mitogenic stimuli from the cell surface to the nucleus. Oncogenically activated raf genes have been shown to encode constitutively active proteins and no longer depend on upstream signals. The goal of this study is to identify upstream activating ligands and downstream substrates of Raf kinase and to elucidate how Raf interacts with them. To these ends we have established several systems expressing wild-type and mutant Raf kinases for in vivo and in vitro analyses. Retroviral and eukaryotic expression vectors will show which mutants have acquired transforming or suppressor activity in vivo, while Raf proteins produced in bacteria or baculovirus expression systems will be used for in vitro reconstitution assays.

Methods Employed:

Molecular cloning and site-directed and linker-insertion mutagenesis were performed following standard protocols. Recombinant baculovirus expressing wild-type and mutant raf genes are grown in Spodoptera frugiperda cells in Grace's medium with fetal calf serum following protocols established by M. Sumners. In vitro transcription and translation was done using commercially available kits following the suppliers' recommendations. Transfection of eukaryotic cells was done by the calcium-phosphate co-precipitation method. Quantitative S1 analysis of RNA was performed according to standard protocols.

Major Findings:

1. Expression of raf oncogenes activates the polyoma enhancer region 1 (PEA1) transcription-factor motif. The PEA1-motif, which is related to the activator protein 1 (AP1) consensus 1, is activated by expression of several oncogenes (v-src, polyoma middle T, ras, v-mos, c-fos) as well as by serum ingredients and the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate (TPA). The PEA1-motif responds only to one component (PEA1) in thymidine-kinase negative mouse L cells--fibroblast extracts, making it likely that PEA1 constitutes one of the final targets of the signal transduction cascade. We tested whether the PEA1-motif can also be activated by Raf. This was done by cotransfecting a plasmid containing the beta-globin gene under the control of the PEA1-motif with different raf expression plasmids. A plasmid with a mutant, unresponsive PEA1-motif was used in control experiments. Cotransfection with v-raf expression plasmids resulted in a tenfold increase of

PEA1-motif controlled transcription, while expression of c-raf did not activate the PEA1-motif. Inactivating mutations in v-raf, like a premature termination codon or a mutation in the ATP-binding site, also abolished PEA1 activation. High levels of wild-type raf expression did not affect the PEA1-motif, while a mutant raf gene, which showed only a low level of oncogenic activity in transformation assays, was able to activate it. Truncated c-raf, which only expresses the Raf kinase domain, is a highly active oncogene, and also showed high levels of PEA1 activation, demonstrating that only the Raf kinase domain is necessary for this activation. Results similar to those obtained with c-raf constructs were obtained when A-raf expression plasmids were used in the experiments. Full-length A-raf did not result in an enhancement of PEA1-controlled transcription, while truncated mouse and human A-raf stimulated PEA1-motif activity.

From other studies we know that several of the oncogenes previously tested for PEA1 activation, especially membrane-associated tyrosine kinases like platelet-derived growth factor (PDGF)-receptor, will tyrosine-phosphorylate Raf protein upon treatment with the appropriate growth factor. The tyrosine phosphorylation is associated with Raf kinase activation. These findings taken together suggest that Raf is a downstream link in the signal cascade and relays the signal from the cell surface to the nuclear factors. We have shown that Raf directly binds to the PDGF receptor in immuno-coprecipitation assays using PDGF-receptor and Raf proteins produced in the baculovirus expression system. Using a similar approach we are currently investigating whether a similar direct relationship exists for AP1 and Raf.

2. The role of functional domains in the interaction of Raf with ligands and substrates. raf proteins belong to the oncogene family of protein kinases and show a distant but clear homology to protein kinase C (PKC). To verify these relationships and learn which positions of the proteins are functionally important for transforming and normal activities, we have generated several mutant raf genes with mutations in possible critical structures. Several of these mutants have scored either as inactivating or activating mutations in transfection/transformation assays. Testing the activity of mutants, especially those that are inactivated in this system, is difficult due to the presence of the constitutively expressed endogenous c-raf gene. To overcome this problem we have transferred several mutant raf genes into a baculovirus expression system and obtained high levels of Raf protein production. The baculovirus system has several advantages over the bacterial expression system; proteins are normally modified and several different viruses carrying genes for various links in the signal transduction pathway can be introduced into a cell at once, allowing for their concomitant expression.

One domain of the protein of particular interest is the serine/threonine and tyrosine kinase substrate site, Tyr-Gly-Arg-Arg-Ala-Ser, found around position 40 in the c-Raf protein. Several lines of evidence suggest that this region is of importance to the regulation of raf kinase activity. PKC has a pseudo-substrate sequence located at its amino-terminal end; oligopeptides carrying this pseudo-substrate were found to inhibit PKC activity. We have found that full-length c-Raf protein, when overexpressed, does not induce cell transformation. However, when expressed as a gag-c-raf fusion, the protein induces cell transformation, suggesting that the configuration of the amino-terminal

end of the Raf protein is important to the regulation of its kinase activity. To test whether this involves the substrate site, we mutated two arginine residues to Ile-Glu and, separately, the serine to alanine. Neither of these mutations on the full-length c-raf gene brought about its oncogenic activation in NIH 3T3 cells. In addition to a possible role of this sequence in the autoregulation of Raf, it is likely that the tyrosine residue is the substrate site for the phosphorylation of Raf by PDGF. To test this we have mutated the tyrosine codon into a tryptophane codon.

Publications:

Benveniste RE, Raben D, Hill RW, Knott WB, Jahrling PB, Arthur LO, Morton WR, Henderson LE, Heidecker G. Molecular characterization and comparison of SIV isolates from macaques, mangabeys, and African monkeys. J Med Primatol (In Press).

Heidecker G, Cleveland JL, Beck T, Huleihel M, Kolch W, Storm SM, Rapp UR. Role of raf and myc oncogenes in signal transduction. In: Colburn N, ed. Genes and signal transduction in multistage carcinogenesis. New York: Marcel Dekker, 1989;339-74.

Rapp UR, Heidecker G, Huleihel M, Cleveland JL, Choi WC, Pawson T, Ihle JN, Anderson WB. Raf family serine/threonine protein kinases in mitogen signal transduction. Cold Spring Harbor Symp Quant Biol 1988;53:173-84.

Wasylyk C, Wasylyk B, Heidecker G, Huleihel M, Rapp U. Expression of raf oncogenes activates the transcription factor PEA1. Mol Cell Biol (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05580-02 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*
Human Genetic Loci Which Influence Susceptibility to HIV Infection and Pathology

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Stephen J. O'Brien	Chief	LVC	NCI
Others:	Raleigh Boaze	Biological Laboratory Technician	LVC	NCI
	James Goedert	Coordinator, AIDS Working Group	EEB	NCI
	Dean Mann	Chief, Immunogenetics Section	LVC	NCI
	William Blattner	Chief, Viral Epidemiology Section	EEB	NCI
	Rashid Aman	Visiting Associate	LVC	NCI

COOPERATING UNITS *(if any)* LCS,NIAAA(D.Goldman);City Clinic Annex,S.F.,CA(G.Rutherford); Dept.Micro.&Immunol.,U.Miami Sch.Med.,FL(W.Parks);AIDSP,NIAID(I.Obrams);Dept. Statistics,NC State U.,Raleigh,NC(B.Weir);Epid.Studies Sect.,CDC,Atlanta,GA (J. Jason);U.Texas,Sch.Pub.Health,Houston,TX(P.Beasley);PRI,Fred.,MD(C.Winkler,M.Dean)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
2.3	0.9	1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The focus of this project is to identify host genetic factors which influence host resistance or susceptibility to infection and disease by two common viruses known to be associated with human neoplasms and/or immunosuppression: HIV and hepatitis B virus (Hep B). The AIDS epidemic has resulted in a massive research effort to understand the epidemiology, molecular biology, and pathology of the etiologic agent, HIV. Hepatocellular carcinoma is a leading neoplasm worldwide and is caused by Hep B. We are attempting to identify genes that influence viral infection and pathology by using restriction fragment length polymorphism (RFLP) markers distributed throughout the genome to detect distortions in population genetic equilibrium in different disease categories. We have identified and acquired approximately 450 clones which detect human polymorphisms at a resolution of 2-10 centiMorgans. In our probes collected, each chromosome is represented by at least 10 loci. We have received 1,066 specimens from different HIV risk groups and established 1,053 lymphoblastoid cell lines. Nearly 1,200 specimens have been collected from Hep B index cases and their first degree relatives. A computer system has been developed to track inventory of biological reagents, to record clinical information and individual genotypes and to complete genetic associations in the study groups. RFLP genotypes are presently being collected at a rate of 1,000 per week. The first total analysis of a study cohort is projected to be complete by Fall 1989.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Stephen J. O'Brien	Chief	LVC	NCI
Raleigh Boaze	Biological Laboratory Technician	LVC	NCI
James Goedert	Coordinator, AIDS Working Group	EEB	NCI
Dean Mann	Chief, Immunogenetics Section	LVC	NCI
William Blattner	Chief, Viral Epidemiology Section	EEB	NCI
Rashid Aman	Visiting Associate	LVC	NCI

Objectives:

A striking feature of human viral infections is the heterogeneity of host response to exposure, infection, and disease development. While this differential response may result from phenotypic differences in the viral pathogen, a second interpretation of the epidemiological data is that there are genetic differences in human populations which impact on susceptibility or resistance to viral pathogens. The objective of this project is to identify those genetic factors which are operative in differential host response to two pathological human viruses, HIV and hepatitis B virus (Hep B), using a combination of molecular biology, population genetic principles, and epidemiology.

Methods Employed:

The following techniques were employed: (1) resolution of human restriction fragment length polymorphism (RFLP) using human DNA clones derived by the human genetics community; (2) immortalization of B-lymphocytes from fresh blood or from cryopreserved peripheral blood leukocytes (PBL) by transformation with Epstein-Barr virus (EBV); (3) immunological assays (ELISA, western blot) for the detection of anti-HIV antibodies and HIV antigens in lymphocytes and sera or lymphoblastoid cell lines (LCL) and their supernatants; (4) standard mathematical methods to detect distortion in genetic equilibria in natural populations; and (5) electrophoretic resolution of polymorphic protein markers resolved using allozymes or two-dimensional electrophoresis (2DE).

Major Findings:

1. Strategy. By analogy to murine and feline retroviral diseases, there are conceivably numerous genetic loci which can influence (positively or negatively) disease susceptibility. Human genetic loci which impact on AIDS sensitivity must also exist. This project represents an attempt to use a combination of available human genetic technology, population genetic principles, and epidemiology to identify those genes in man. The strategy follows: The human gene map has grown in the last decade to include over 5,000 genes. Over 3,000 are defined by molecular clones and over 1,500 of these genes are polymorphic for RFLPs. In addition, nearly 50 polymorphic loci for proteins (resolved by isozyme and 2DE gels) have been reported. Thus, it is possible to take the human gene map and to identify abundantly

polymorphic loci at an average of 5- to 10-centiMorgan (cM) units along every chromosome (from 1 to X). The project involves collection of blood from AIDS cohorts in several locales with large numbers of patients in each disease category (e.g., antibody positive for ≥3 years and asymptomatic) plus matched control populations. Patient B-cells will be transformed with EBV and expanded for DNA extraction. Distortion of three population genetic parameters of polymorphic loci (allelic frequency, Hardy-Weinberg equilibrium, and linkage equilibria of paired loci) would be interpreted as a signal for the occurrence of genes which impact on a patient's occurrence in a particular disease category. The gene markers would include polymorphic DNA segments, allozymes, and 2DE variants. Included in this panel would be clones of candidate loci such as the T-cell receptor, HLA, IgG, CD4, IL2R, etc. We would take advantage of the thorough serological workups on human AIDS patients by also noting exposure, symptoms, and presence of viruses for other diseases, such as cytomegalovirus, HTLV-I, Hep B, and herpesvirus. The wealth of epidemiology background would be considered throughout in defining new disease categories for gene identification.

2. HIV study groups. Collaborations for the HIV study have been established with epidemiologists with well-established cohorts of homosexual men and with the Multicenter AIDS Cohort (MAC) study. At this time, almost 1,200 hemophiliacs have been identified and will participate in this study. Emphasis is being placed on individuals who have received HIV contaminated lots of clotting factors and on sibling brothers who are discordant for HIV infection or disease. We are also collaborating with clinicians who have established cohorts of offspring of HIV-positive mothers. A total of 5,991 individuals are participating in this study and nearly 1,100 LCL have been developed. For a detailed listing of collaborators and study groups, see Tables 1-3.

In addition to establishing an LVC repository, red blood cell plasma and PBL specimens are also collected from most patients. Supernatants from LCL frequently contain antibodies against HIV gene products. Thirty LCL have been identified which produce antibodies against one or more gene products of gag, env, or pol by immunoblot assays. Procedures to stabilize antibody production of these potentially valuable diagnostic and research reagents are being developed.

3. Hep B study groups. The hepatitis B study in collaboration with the American University Research Center in Taipei, Taiwan, and Dr. Palmer Beasley is part of a prospective study identifying the Hep B virus as the primary causative agent of hepatocellular carcinoma (HCC). Four study groups have been established: (a) Responders and non-responders to both plasma-derived and recombinant HbsAg vaccine given at birth, 1 month, and 3 months; approximately 90% of the infants develop antibody while 10% do not. (b) HbsAg-positive carriers who either develop cirrhosis or HCC or remain clinically disease-free. (c) Children of highly infectious carrier mothers who either become carriers (90%) or clear the Hep B virus (10%). For each disease/risk category, blood is collected from the index case, two parents, and siblings. We have received 1,600 samples in the form of cryopreserved PBLs from 400 families. To date, 50 LCL's have been established for this cohort.

4. Inventory and data analysis. The project is supported by two IBM AT's and a MacIntosh computer and developed software. Inventories of LCL and DNA probes are managed on dBase III programs. The cell line program tracks samples received, progress of the cultures and the storage of samples. The plasmid data base contains information on each RFLP, including chromosomal location, structure of the construct, and the size and frequency of the alleles detected by each probe. In addition, a diagram of each chromosome has been constructed showing the location of each probe on both a physical and a linkage map. A program to manage the collection of data is being written using the DBase III data base program. This program will be used to record the genotype of each patient generated with the RFLP probes and will allow this information to be interacted with patient information data bases generated by the collaborators. In addition, our terminal is linked to the VAX computer system at the Frederick Cancer Research Facility and to the Human Gene Mapping Library at Yale. The Yale library maintains an updated data base of all known human cloned genes and RFLP probes.

5. Two-dimensional gel electrophoresis. We have successfully completed the 2D gel electrophoresis of lymphocyte samples of individuals from nine three-generation pedigrees. These pedigrees will be used to map polymorphic proteins to individual human chromosomes, and provide a genetic location for these markers. A system to produce a duplicate of silver-stained gels on x-ray film has been developed, and data analysis and storage is being performed in collaboration with Dr. David Goldman, National Institute on Alcohol Abuse and Alcoholism.

6. Collection of human RFLP probes. To date we have collected 450 clones which detect human DNA polymorphisms. Each chromosome is represented by at least ten probes, and the collection covers over 90% of the human genome at a resolution of 10 cM. The majority of these clones (400) have been grown in bulk and are stored in duplicate as both a glycerol stock of transformed bacteria and as DNA. We have tested 150 of these clones, confirming their detection of RFLPs.

7. Detection of new polymorphisms. We have discovered informative RFLPs for several candidate genes which we feel will be useful to our study. These include the gene for the CD4 molecule, the oncogenes jun, ovc-2, and c-raf-1 (also linked to Von Hippell-Lindau's disease); the interleukin-3 (IL-3), GM-CSF, and IL-1 growth factors; the retinoic acid receptor gene, the T-cell receptor zeta gene; and several probes tightly linked to the cystic fibrosis (CF) gene. Two of these polymorphisms are of the variable number of tandem repeat (VNTR) class (IL-1 and zeta). VNTRs show extremely high levels of heterozygosity, and are very useful genetic markers. We have also begun to receive DNA from the Centre D'Étude du Polymorphisme Humain-Human Polymorphism Study Center (CEPH) panel of 40 large human pedigrees. Analysis of these families will provide a genetic location for newly isolated genes.

8. Development of PCR (polymerase chain research) methods for high-resolution detection of cryptic polymorphism in HIV disease associated with human loci. We are utilizing the PCR technique to search for polymorphisms in a number of human genes which are candidate loci for affecting retroviral pathology. In one approach we are amplifying genomic sequences of ~1 kb, and where possible

these regions are designed to include, in addition to coding sequences, either 5′ untranslated, 3′ untranslated, or intronic regions so as to maximize the likelihood of detecting polymorphisms. The PCR products are then analyzed after digestion with frequently cutting restriction enzymes (4-base cutters) on polyacrylamide gels. High resolution analysis following digestion of PCR products can be achieved on sequencing gels when radiolabeled deoxynucleotides (either ^{32}P-dCTP or ^{35}S-dATP) are included during the amplification reaction. Candidate genes involved in interactions with infectious agents (e.g., CD4, T-cell receptor), as well as other genes for which no polymorphisms have been reported, are being analyzed by this technique.

In another approach, we are extending recent developments in the application of PCR technology to detect nucleotide substitution within the HLA-D locus. Using primers for polymorphic regions of the DRβ, DQα, and DQβ loci, target sequences of 200-300 b.p. can be amplified and analyzed by allele-specific oligonucleotide hybridization or by direct sequencing. By these means we hope to generate information that could be useful in disease-association studies and comparisons of haplotypes in various ethnic groups.

9. Linkage disequilibrium in the CF locus. Several RFLP probes tightly linked to the CF gene show significant levels of linkage disequilibrium both with the disease and with each other. As a model system for the use of linkage disequilibrium in human populations, we have examined the degree of disequilibrium between newly discovered RFLPs in this locus in collaboration with Dr. Francis Collins at the University of Michigan. Results show that a group of polymorphisms spanning 300 kb surrounding the pJ3.11 probe show very high levels of disequilibrium in a group of unrelated Caucasians. This result suggests that large regions of DNA displaying significant disequilibrium are likely to exist in the human genome.

Publications:

Dean M. Molecular and genetic analysis of cystic fibrosis. Genomics 1988;3: 93-9.

Iannuzzi MC, Dean M, Drumm ML, Hidaka N, Cole JF, Perry A, Stewart C, Gerrard B, Collins FS. Isolation of additional polymorphic clones from the cystic fibrosis region, using chromosome jumping from D7S8. Am J Hum Genet 1989;44:695-703.

Mann DL, Gilbert DA, Reid Y, Popovic M, Read-Connole E, Gallo RC, Gazdar AF, O'Brien SJ. On the origin of the HIV susceptible human CD4+ cell line H9. AIDS Res (In Press).

O'Brien SJ, Evermann JF. The interface of epidemiology and genetic diversity in free-ranging animal populations. Trends Ecol Evol 1988;3:254-9.

Table 1

Status May 1, 1989
HOMOSEXUAL STUDY GROUPS

Code	Disease Category	Number Projected	Number Received	Number LCL	Number DNA	Location	Collaborators
DGE	Homosexual men	300	240	123	93	New York City Washington, DC	J. Goedert, R. Bigger, and W. Blattner (NCI)
MAC	Kaposi's Sarcoma	60	---	---	---	Multicenter AIDS Cohort (MAC) (Johns Hopkins)	J. Farr, R. Detels, C. Rinaldo, A. Saah, Coordinator: A Munoz
	HIV^+ asymptomatic matched controls	149	---	---	---		
	Pneumocystis carnii pneumonia	101	---	---	---		
	HIV^+ asymptomatic matched controls	101	---	---	---		
	Sum	411	368	271	125		
SFC	HIV (+) Asymptomatic	200	---	---	---	San Francisco	G. Rutherford, A. Lifson, P. O'Malley, (S. F. City Clinic)
	HIV (-) Exposed	200	---	---	---		
	HIV (+) ARC/AIDS	200	---	---	---		
	Sum	600	56	54			
USF	ARC/AIDS	80	4	4	0	San Francisco	J. Zeigler (U.C.S.F.)
UCD	ARC/AIDS	200	0	---	---	Davis	M. Gardner (U.C. Davis)
	Total	1,591	668	442	218		

Z01CP05580-02 LVC

Table 2

Status May 1, 1989
HETEROSEXUAL STUDY GROUPS

Code	Disease Category	Number Projected	Number Received	Number LCL	Number DNA	Location	Collaborators
---	Neonates: HIV (+)	800	0	---	---	Multicenter	J. Pitt (Columbia)
MAI	Neonates: HIV (+)	200	20	18	15	Miami (U. of Miami)	W. Parks
NJD	Parenteral Drug User	---	278	229	229	New York City	J. Goedert, R. Bigger, and W. Blattner (NCI)
DES	HIV (+) Cases with Matched Controls	500	0	---	---	Dar es Salaam Tanzania	S. Maselle (Muhimbili Medical Center)
NAI	HIV (+) Cases with Matched Controls	500	0	---	---	4 locales Kenya	D. Koech (Kenya Medical Research Inst.)
GAB	Prostitutes	500	0	---	---	West Africa Gabon	M. Potts (Family Health International)
	Total	2,500	298	247	244		

Z01CP05580-02 LVC

Table 3

Status May 1, 1989
HEMOPHILIAC STUDY GROUPS

Code	Number Risk Group	Number Projected	Number Received	Number LCL	Number DNA	Location	Collaborators
HEA	Hemophiliacs	1,200	32	28	21	Multicenter	J. Goedert, R. Bigger, and W. Blattner (NCI)
HEAS	Hemophiliac SIB Sets	100	---	---	---	Multicenter	As above
HEAP	Hemophiliac Spouses	200	---	---	---	Multicenter	As above
HCD	Hemophiliacs, HIV Defined Lots	200	18	8	---	Multicenter	J. Jason (CDC)
PAN	Pediatrics	200	---	---	---	Los Angeles (Children's Hospital)	E. Gomperts
Total		1,900	50	36	21		

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05581-02 LVC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less Title must fit on one line between the borders.)*

Role of Kinase Oncogenes in Growth Factor Abrogation and c-myc Regulation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Ulf R. Rapp Chief, Viral Pathology Section LVC NCI

Others: None

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (M. Dean, P. Lloyd); Univ. of California at San Diego, La Jolla, CA (J. Wang); St. Jude's Children's Hospital, Memphis, TN (J. Cleveland); Johns Hopkins University, Baltimore, MD (S. May)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have investigated the functional significance of various oncogenes in interleukin-3 (IL-3) signal transduction in two myeloid-dependent cell lines--FCD-P1 and 32DC13, by testing their potential to abrogate IL-3 requirements and to induce the expression of genes which are normally regulated by IL-3. In these cells, IL-3 tightly regulates expression of c-myc, c-fos, and junB. We and others have demonstrated conditional abrogation of IL-3 dependence by introduction of temperature-sensitive (ts) v-abl retroviral constructs. Furthermore, this tyrosine kinase mimics IL-3 in its ability to regulate c-myc transcription, yet differs from IL-3 signal transduction in that ts v-abl fails to induce c-fos and junB, suggesting that IL-3 induction of these two genes requires distinct signalling pathways. In comparison to abrogation by tyrosine kinase oncogenes, we have demonstrated that exogenous v-myc or c-myc can either completely or partially abrogate IL-3 requirements, respectively. In contrast, v-raf is even less efficient than c-myc in abrogating IL-3 requirements. However, when constructs carrying both raf plus myc oncogenes are introduced into IL-3-dependent lines or if raf or myc viruses are used to superinfect raf- or myc-containing factor-dependent clones, factor-independent lines are generated with frequencies comparable to tyrosine kinase oncogene constructs. The mechanism for this synergism appears to be the result of combination of two jointly required pathways which function in IL-3 signal transduction. This hypothesis is based upon the finding that introduction of exogenous, activated v-raf has no effect upon c-myc, c-fos, and junB expression. To examine other potential downstream intermediates in IL-3 signal transduction, the effects of protein kinase C constructs upon the regulation of cell growth and gene expression, and its potential to synergize with other oncogenes will also be presented.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp Chief, Viral Pathology Section LVC NCI

Objectives:

The general objective of this study is to identify the second messengers involved in growth factor signal transduction and regulation of c-myc in interleukin-3 (IL-3)-dependent murine myeloid cells and in NIH 3T3 fibroblastic cells. Specifically, we will determine (1) candidate genes involved in growth factor signal transduction by introducing a variety of oncogenes into these cells and testing their ability to abrogate growth factor requirements, (2) whether regulation of c-myc is affected by these exogenous oncogenes and what the significance of this regulation is, (3) how expression of c-myc is regulated in IL-3 signal transduction and in cells abrogated of growth factor requirements by introduction of exogenous oncogenes and what targets on the c-myc gene are responsive to this regulation, and (4) the cascade of second messengers acting downstream in both ligand/receptor signal transduction and in signalling from oncogenes which abrogate factor dependence. Major emphasis will be on activation of raf and on the protein kinase C (PKC)-family of protein kinases.

Methods Employed:

Recombinant retroviruses containing various oncogenes were constructed using standard recombinant DNA technology. High-titer stocks of these viruses pseudotyped with various helper viruses were prepared from transfected NIH 3T3 cells using established techniques. RNA and DNA blot analyses, S1 nuclease and RNase A mapping of transcripts, and nuclear run-on assays are performed using standard protocols.

Major Findings:

The data described in this report demonstrate, for the first time, that tyrosine kinase oncogenes can trans-activate expression of the c-myc proto-oncogene. Combined with our previous findings showing that addition of IL-3 rapidly induces both tyrosine phosphorylation and c-myc expression, the experiments described here demonstrate a direct physiologic link between these two events in IL-3 signal transduction. The major findings are as follows:

1. Introduction of tyrosine kinase oncogenes (including v-abl, v-src, v-fms, and trk) acutely abrogate IL-3 dependence of FDC-P1 cells at very high frequencies (4-5 logs higher than with control neo viruses), suggesting that factor abrogation is not likely to require other secondary events. Using conditional temperature-sensitive (ts) abl retroviruses we demonstrated that, at least for this tyrosine kinase oncogene, factor abrogation requires the presence of functional abl protein. In contrast, raf family serine/threonine kinase oncogenes were inefficient in abrogating IL-3 requirements (only a

tenfold increase in frequency) of FDC-P1 cells, but did relieve the NIH 3T3 cells of their serum growth factor requirements, suggesting different signalling pathways in the two different cell types.

2. FDC-P1 cells which have been abrogated of their IL-3 requirements by introduction of tyrosine kinase oncogenes all constitutively express c-myc in the absence of IL-3, whereas in normal FDC-P1 cells, c-myc expression strictly requires the presence of this ligand. Therefore, although tyrosine kinase oncogenes likely perform several functions that are jointly involved in growth factor abrogation, one essential activity which they share for IL-3 abrogation is their ability to induce c-myc. The constitutive expression of c-myc in these cells was not due to any gross alterations or proviral insertions, as determined by Southern blot analyses, nor was it due to alternative promoter usage or to mutations in c-myc exon 1, which has been shown to harbor sites required for an attenuation regulation of c-myc transcription.

3. Using viruses expressing wild-type and ts versions of the abl tyrosine protein kinase, we have demonstrated first that constitutive expression of c-myc requires the presence of functional abl protein and second, using temperature shift experiments, that abl tyrosine protein kinase trans-activates expression of c-myc mRNA.

4. A potential synergistic effect on abrogation of IL-3 dependence by v-myc was tested. Combination of these oncogenes was observed to bypass IL-3 signal transduction with an efficiency comparable to those of tyrosine kinase oncogenes. These findings are consistent with a model for growth regulation by tyrosine kinase class receptors, which involves activation of, minimally, two jointly required pathways for mitogen signal transduction--one involving PKC and another in which raf protein kinase functions

5. Activation of raf-1 protein kinase by IL-3 was analyzed using FDC-P1 myeloid cells. IL-3 was found to regulate raf kinase phosphorylation and increased in specific activity. This activation of raf-1 by PKC appears to be essential for growth regulation of FDC-P1 cells by IL-3 since raf-specific antisense RNA blocks IL-3 mitogenesis.

Publications:

Cleveland JL, Dean M, Wang JY, Hedge A-M, Ihle JN, Rapp UR. Abrogation of IL-3 dependence of myeloid FDC-P1 cells by tyrosine kinase oncogenes is associated with induction of c-myc. In: Melchers F, Potter M, eds. Current topics in microbiology and immunology. New York: Springer-Verlag, 1988;300-9.

Dean M, Cleveland J, Kim H-Y, Campisi J, Levine RA, Ihle J, Rapp U. Deregulation of the c-myc and N-myc genes in transformed cells. In: Melchers F, Potter M, eds. Current topics in microbiology and immunology. New York: Springer-Verlag, 1988;216-22.

Rapp UR, Heidecker G, Huleihel M, Cleveland JL, Choi WC, Pawson T, Ihle JN, Anderson WB. raf family serine/threonine/protein kinases in mitogen signal transduction. Cold Spring Harbor Symp Quan Biol 1988;53:173-84.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05582-02 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Growth Modulation and Analysis of Chemically-Induced Tumors

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Others:	Stephen M. Storm	Biologist	LVC	NCI

COOPERATING UNITS (if any)
St. Jude's Children's Hospital, Memphis, TN (J.L. Cleveland); Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, NIH (B. Moss)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Viral Pathology Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.8	0.2	0.6

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We have established a mouse model system for the rapid induction of lung adenocarcinomas and lymphomas in order to investigate lung carcinogenesis in vivo, and to examine potential regimens for growth modulation of these tumors. A transplacental injection of 1-ethyl-1-nitrosourea at day 16 of gestation, followed by promotion with butylated hydroxytoluene beginning 5 weeks after birth, results in 90% of animals developing tumors within 5 to 14 weeks of age. Both tumor types contain transforming DNA as defined by NIH 3T3 cell transformation assays. We are currently investigating the nature of this transforming DNA through several means. Although raf does not appear to be the transforming gene in NIH 3T3 cell assays, high levels of normal-sized c-raf-1 are expressed in both tumors and cell lines as determined by Northern and Western blotting, consistent with a role for raf in the development and/or maintenance of these tumors.

raf protein vaccinations administered at 3, 4, and 5 weeks of age apparently eliminate the promoted phase of tumor growth. Since constitutive presence of oncogene proteins as anti-tumor antigens may provide even more effective protection, we have developed a set of raf-carrying vaccinia virus vectors for use as vaccines, including a v-raf construct with a point mutation that eliminates its transforming activity. These constructs are being tested for their tumor growth modulating ability. In addition, we intend to generate vaccinia viruses expressing other oncoproteins which may play a role in the generation or maintenance of these tumors for use as vaccines. We are also infecting Balb/3T3 cells with the v-raf mutant for use in in vivo T-cell recognition assays in Balb mice in order to delineate immune responses responsible for the protection afforded by raf protein vaccinations.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Stephen M. Storm	Biologist	LVC	NCI

Objectives:

To employ an animal model system which we have developed for the induction of lung carcinomas relevant to human lung cancer in order to define regimens for the prevention and/or reversal of neoplasms involving raf and other oncogenes. To carry out molecular analysis of tumors in order to identify potential neoplastic mutations, enabling us to devise more effective treatment procedures.

Methods Employed:

For induction of lymphomas and lung adenocarcinomas in mice, pregnant females were injected transplacentally with ethylnitrosourea (ENU). To accelerate tumor development in the offspring, weanling age F_1 mice were promoted with weekly injections of butylated hydroxytoluene (BHT). Vaccination of newborn mice with oncogene protein followed procedures previously developed for vaccination of high leukemia strains of mice with retroviral structural proteins. Oncogene-expressing recombinant vaccinia viruses were generated by standard procedures for in vivo recombination between vaccinia virus and plasmids. raf-expressing vaccinia viruses are administered to carcinogen-treated animals via tail scratch. Molecular analysis of tumors was per standard procedures for DNA (Southern), RNA (Northern) and protein (Western analysis). In addition, tumor-derived DNA and RNA are being screened for potential mutations via RNase protection assays and the polymerase chain reaction (PCR).

Major Findings:

Tumor induction experiments, in which varying amounts of carcinogen (ENU) and promoter (BHT) were administered, determined the dosages for maximal tumor induction. This is of importance in testing the efficacy of various treatment regimens as the protective effect of purified raf protein vaccination was seen only in animals subjected to a schedule of very rapid tumor induction. Generation of monoclonal antibodies against the purified 30-kD v-raf protein used in earlier vaccination experiments showed that it is indeed immunogenic in these animals. To test the effectiveness of constitutively expressed oncoprotein as a vaccine in our system, v-raf vaccinia viruses were constructed and administered to carcinogen-treated animals. These experiments are currently underway. In addition, we plan to test vaccinia viruses expressing other oncogenes for their ability to affect tumor growth modulation in their host. Transformed cell lines from both T-cell lymphomas and lung adenocarcinomas were generated by transfection of tumor DNA into NIH 3T3 cells

and also by culturing of primary tumors. Tumors and tumor-derived cell lines show no evidence of rearranged oncogenes at the level of Northern and Southern blotting for approximately 20 proto-oncogenes tested to date. Many of the proto-oncogenes tested so far are expressed in higher levels in tumor than in control tissue; however, none of them show a uniform high level in all tumors as does c-raf-1. We are in the process of examining expression levels and looking for rearrangements of other proto-oncogenes. One chemically-induced T-cell lymphoma showed evidence of a K-ras mutation at codon 12, but none of the other lymphomas (12), lung adenocarcinomas (17), or cell lines (10) tested were positive for a mutation at this site as determined by RNase protection assays, suggesting that this may have been a secondary event. PCR-amplified DNA from both lung adenocarcinomas and lymphomas was cloned and sequenced at codons 12 and 13 of H-ras, K-ras, and N-ras. In no instance was a mutation detected. We are now examining codon 61 of these genes for mutations. We have also checked the 5′ end of c-raf transcripts for point mutations in both tumor types by RNase protection assays and none was detected. Analysis of the 3′ half is currently being performed. Since all of these tumors were generated in the same way, we feel that identification of the transforming sequence(s) will allow us to tailor an even more effective vaccination protocol, and give further insight into the role raf may be playing in these tumors.

Publications:

Giardina SL, Storm SM, Longo DL, Mathieson BJ, Rapp UR, Varesio L. Characterization of a murine monoclonal antibody that detects a C-terminal fragment of the raf oncogene product. J Immunol 1988;140:3528-33.

Rapp UR, Cleveland JL, Bonner TI, Storm SM. The raf oncogenes. In: Reddy EP, ed. The oncogene handbook. Copenhagen: Elsevier Science Publishers, 1988;213-53.

Rapp UR, Huleihel M, Pawson T, Linnoila I, Minna JD, Heidecker G, Cleveland JL, Beck T, Forchhammer J, Storm SM. Role of raf genes in lung carcinogenesis. In: Hansen HH, ed. Proceedings from the international conference on hormones, growth factors and oncogenes in pulmonary carcinoma. Copenhagen: Elsevier Science Publishers (In Press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05583-02 LVC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 charecters or less. Title must fit on one line between the borders.)*

Regulation of Equine Infectious Anemia Virus Gene Expression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: David Derse Senior Staff Fellow LVC NCI

Others: None

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (P. Dorn)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.4	0.2	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This laboratory previously reported that the equine infectious anemia virus (EIAV) promoter is specifically activated in virus-infected cells and that this activation requires both a cis-acting element located in the proximity of the RNA start site and a virus-encoded trans-acting factor. The trans-acting factor or tat gene and its protein product have now been examined in greater detail. A DNA fragment from the central region of the genome was shown to activate the EIAV promoter in trans when expressed in cells. Deletions and site-directed mutagenesis of this DNA fragment revealed that the active tat exon is contained in a 50-codon open reading frame (ORF) previously termed S1. Activation by EIAV tat was accompanied by increases in steady state levels of RNA directed by the EIAV promoter. A cDNA library was constructed from a productively infected cell line and screened with a probe representing the active tat exon. Several positive clones were isolated and subcloned for nucleotide sequencing and to test function in transfected cells. Nucleotide sequence of the tat gene showed that the multiply spliced message is formed by joining regions from the 5′ end of the virus, the central region (S1 ORF), and the 3′ end of the genome. The deduced tat amino acid sequence shows several domains that are closely related to the tat proteins of the human and simian immunodeficiency viruses. The EIAV tat cDNAs were functional since their expression in cells resulted in activation of the EIAV promoter. The tat gene is currently being altered in vitro to define amino acids essential for its activity and specificity.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

David Derse Senior Staff Fellow LVC NCI

Objectives:

1. Characterize the cis- and trans-acting components of the regulatory apparatus that controls the equine infectious anemia virus (EIAV) gene expression.

2. Construct a cDNA library representing EIAV RNAs to determine the nucleotide sequence of the tat message, deduce the amino acid sequence of the tat protein, and define the splicing pattern used to generate the tat mRNA.

3. Test the functional activities of the tat cDNAs by expression in mammalian cells.

4. Examine the structure-function relationships in the tat protein by introducing missense mutations by site-directed mutagenesis.

Methods Employed:

The following methods were employed: (1) cDNA cloning, (2) nucleotide sequencing, (3) recombinant plasmid construction, (4) site-directed mutagenesis, (5) transfection of mammalian cells, and (6) Northern blotting and chloroamphenicol acetyl transferase (CAT) assays.

Major Findings:

1. The active EIAV tat exon is located in the central region of the virus. A proviral DNA fragment encompassing the region from the 3′ end of the pol gene through the 5′ end of the env gene was inserted into a eukaryotic expression vector and transfected into cells in combination with plasmids that contain the CAT gene controlled by the EIAV promoter. Expression of this fragment was accompanied by increased levels of RNA directed by the EIAV promoter and required cis-acting sequences in the proximity of the RNA start site in the EIAV long terminal repeat. Deletion and frameshift mutations within the putative tat fragment identified a 50-codon open reading frame (ORF), previously designated S1, as the tat gene.

2. Nucleotide and deduced amino acid sequence of EIAV tat cDNAs. A cDNA library was constructed using poly(A)+ RNA from the productively infected cell line, E-FEA. Clones that hybridized to a probe representing the active tat exon were isolated and characterized. Several clones were sequenced revealing that the tat message is generated by splicing three exons derived from the 5′ end, middle, and 3′ end of the virus. There is a stop codon at the end of the central exon followed by another extended ORF; this latter frame may encode

another regulatory protein in a manner analogous to other lentiviruses. The deduced amino acid sequence of EIAV tat revealed a protein with two domains very similar to regions of HIV and simian immunodeficiency virus tat proteins; however, the EIAV protein lacks a domain shared by the primate virus proteins but has been shown to be dispensable for transactivation. Both the cDNA and genomic EIAV tat sequences lack an AUG initiation codon and may use an alternative initiator.

3. Functional activity of tat cDNAs. The tat cDNA clones were inserted into eukaryotic expression vectors and transfected into a variety of mammalian cell lines to examine activity. Expression of these plasmids resulted in the activation of the EIAV promoter thus demonstrating their competence. The mechanism of translation initiation is currently being studied. In addition, the contribution of various amino acids to the activity and specificity of EIAV tat is being examined by in vitro mutagenesis of the protein.

Publications:

Dorn PL, Derse D. Cis- and trans-acting regulation of gene expression of equine infectious anemia virus. J Virol 1988;62:3522-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05584-02 LVC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Genomic Organization in Nonhuman Primates and Other Comparative Genetic Studies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Hector N. Seuanez	Visiting Scientist	LVC	NCI
Others:	Mary A. Eichelberger	Microbiologist	LVC	NCI
	Stephen J. O'Brien	Chief	LVC	NCI

COOPERATING UNITS *(if any)*

Program Resources, Inc., Frederick, MD (W. Modi, K. Richards)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Genetics Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.0	0.6	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Nonhuman primates are frequently used in several areas of biomedical research such as neurobiology, reproductive physiology, infectious diseases, immunology and cancer research. Despite their frequent utilization, however, several primate species require a genetic characterization for the establishment of adequate comparisons with the human and for their standardization as reliable animal models.

The study of the genome organization of the nonhuman primates has been approached by karyological studies of genera in which fragmentary data are available. These genera, belonging to the neotropical family of Callitrichid monkeys (marmosets) are widespread in the wild and frequently captive bred in colonies, though their genetic characterization is presently incomplete. Moreover, very limited data are presently available on gene assignment in the nonhuman primates. Comparative gene maps are available for only 12 species in which the number of mapped genes ranges from a minimum of 25 to a maximum of 65. This contrasts strikingly with the human in which the known number of structural loci and anonymous gene sequences amounts to some 5000 markers. For this reason, a hybrid cell panel has been constructed for the New World spider monkey species, Ateles paniscus (2n = 34) using a rodent receptor cell line and a donor primate fibroblast cell line. Approximately 70 hybrid cell lines have been cloned in selective medium and analyzed by electrophoresis for 25-30 gene products. A preliminary analysis of these results has allowed for the identification of presumptive syntenic groups in this species. A cell hybridization experiment with rodent X xenopus produced several stable hybrids now under genetic analysis.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Hector N. Seuanez	Visiting Scientist	LVC	NCI
Mary A. Eichelberger	Microbiologist	LVC	NCI
Stephen J. O'Brien	Chief	LVC	NCI

Objectives:

The specific objectives of this project are (1) establishment of fibroblast cell lines from approximately 15 species of New World monkeys; (2) comparison of chromosomal banding patterns among these monkey species; (3) gene assignment in Ateles paniscus by somatic cell hybridization; (4) comparison of linkage group conservation of Xenopus with mammals, birds, and fish; (5) generation of a fundamental genetic foundation for the study of molecular embryogenesis in Xenopus; (6) attempt to identify heteromorphic sex chromosomes and to address the question of whether or not dosage compensation occurs in amphibians; and (7) examination of the organization and syntenic relationships of duplicate loci in the tetraploid, Xenopus laevis.

Methods Employed:

The following techniques are being utilized: (1) primary fibroblasts are obtained by cultivating skin biopsies in tissue culture medium, (2) somatic cell fusion and tissue culture propagation of cell hybrids, (3) cytogenic analysis of metaphase chromosomes, (4) protein starch gel electrophoresis, and (5) Southern blot and DNA filter hybridization.

Major Findings:

1. Comparative chromosome morphology in three Callitrichid genera: Cebuella, Callithrix, and Leontopithecus. A G-band karyotypic analysis was carried out in individual species groups of three Callitrichid primate genera: Cebuella, Callithrix, and Leontopithecus. Within Callithrix, the karyotypes of the morphologically distinct and geographically isolated morphotypes, C. jacchus jacchus and C. jacchus penicillata, were identical. Within the lion tamarin genus, Leontopithecus, the karyotypes of the three morphotypes (L. rosalia rosalia, L. rosalia chrysomelas and L. rosalia chrysopygus) were also indistinguishable from one another. These results are consistent with the taxonomic designation of subspecies rank to the different morphotypes. A comparison of type specimens among the three Callitrichid genera showed that their phyletic radiation has been paralleled by a limited number of chromosome rearrangements and a relatively high amount of karyotypic invariance. A fusion/fission event has been postulated to account for the difference in diploid number between Cebuella (2n = 44) and the other species (2n = 46). The karyotype of Callithrix jacchus was found to be more directly derived from Cebuella than was that of Leontopithecus. These findings differ from the previous proposition that Leontopithecus might have diverged from a common Callitrichid ancestor before the emergence of the genus Callithrix.

2. Chromosome banding comparisons of Callimico goeldii and its position within the Platyrrhine suborder. These studies have shown that C. goelddi should be included within the family Callitrichidae, as indicated by a parsimonious reconstruction of chromosome phylogenies between Callimico, Cebus apella, and Callithrix jacchus.

3. Identification of the pattern of late DNA replication in the allocyclic X chromosome in Cebus apella and Leontopithecus rosalia. Studies of late DNA replication, using the thymidine analogue 5-bromodeoxyuridine, have shown that the allocyclic X chromosome of human females shows a predominant lymphocyte pattern and a fibroblast pattern. Our studies in Cebus and in Leontopithecus have shown that the allocyclic X chromosomes of these species show a single pattern of late DNA replication, both in lymphocytes and fibroblasts, which is similar to the one found in human fibroblasts.

4. An analysis of syntenic associations in the neotropical monkey species Ateles paniscus, based on some 70 clones and 40 subclones has shown the following syntenic arrangements: PGD-ME1-AK1, MDH1-GOT2-DIA4, SOD1-SOD2, PEP-NP-MPI-HEXA, HPRT-G6PD, ACP2-LDH1, IDH2, MDH2, GUSB, GPI, ESD, and ADA. Chromosome analysis in 10 hybrid cell clones has been completed and will be extended to at least 40 clones before chromosome analyses are made. Data on gene associations in this species shows that some human syntenic groups have been conserved, as is the case of HPRT-G6PD (X), GOT2-DIA4 (16), ACP2-LDHA (11), MPI-HEXA (15). However, the chromosome complement of Ateles paniscus chamek (2n = 34) and man (2n = 46) cannot be simply derived from one another due to extensive chromosome rearrangement during phyletic divergence. Thus, the disruption of some human syntenic groups or the appearance of new associations is a logical consequence of this process. Examples of dissociations are: ME1-SOD2 (human 6), MDH2-GUSB (human 7), IDH2-and MPI/HEXA (human 15), APRT and DIA4/GOT2 (human 16). Examples of new associations are: PGD-ME1-AK1 (human 1p+6+9), MDH1-GOT2-DIA4 (human 2p+16), SOD1-SOD2 (human 21+6), PEP-NP-MPI-HEXA (human 12+14+15).

5. Development and characterization of a panel of rodent X Xenopus somatic cell hybrids. Somatic cell fusion was carried out between Xenopus erythrocytes and fibroblast cells of four different mutant rodent cell lines: RAG (mouse, $HPRT^-$); LM (mouse, TK^-); E36 (hamster, $HPRT^-$); and BHK (hamster, TK^-). Viable hybrids result from the RAG and BHK fusions. Approximately 20 such hybrids were expanded in tissue culture and cryogenically frozen. Using biotinylated Xenopus genomic DNA as a hybridization probe, the chromosomal composition of the hybrids could be determined. Results indicate that interspecific translocations are fairly common in some cell lines but are apparently absent in others. A fair number (approximately 50) of complementary DNA clones from Xenopus have been obtained in different laboratories around the world. Efforts are currently underway to contact collaborators who will make these clones available for mapping purposes. Genomic DNAs have been prepared from Xenopus liver, rodent parental cell lines, and hybrids and await preliminary hybridization analyses.

Publications:

Seuanez HN, Forman L, Alves G. Comparative chromosome morphology in three Callitrichid genera: Cebuella, Callithrix, and Leontopithecus. 'J Hered 1988;79:418-24.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05618-01 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Construction of a Novel Class of Retroviral Vector Using BLV and HTLV-I

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: David Derse Senior Staff Fellow LVC NCI

Others: None

COOPERATING UNITS *(if any)*
Program Resources, Inc., Frederick, MD (L. Martarano)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Genetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Bovine leukemia virus (BLV) and human T-cell leukemia virus type I (HTLV-I) were used as the basis of a new type of "activator-dependent" retroviral vector. The feasibility of this approach was demonstrated by replacing the tax/rex genes of BLV with the bacterial neomycin resistance gene controlled by the SV40 promoter; in all other respects the virus was competent and is designated BLV-SVNEO. Transfection of cells with plasmids that contain BLV-SVNEO resulted in the constitutive expression of the neo gene. In contrast, BLV genes were expressed only when both BLV tax and rex were supplied in trans. The release of infectious BLV-SVNEO after transfection of cells with BLV-SVNEO and tax/rex expression plasmids was reflected in the number of G418-resistant colonies that developed after exposure of susceptible cells to the filtered culture medium. Both tax and rex were required in trans for the production and release of BLV-SVNEO. A variety of cell lines were susceptible to infection with BLV-SVNEO including those of bovine, human, canine, feline, and murine origin. BLV-producing cell lines were resistant to superinfection with BLV-SVNEO suggesting that this is a receptor-mediated infection. A similar recombinant retrovirus was constructed from HTLV-I; in this case the tax/rex genes and part of the env gene were replaced with the neo gene controlled by a cytomegalovirus promoter. Production of infectious virus here requires complementation with tax, rex, and env. These experiments demonstrate the potential utility of these vectors in gene delivery. In addition, they should prove useful in themselves as a means to quantify virus infectivity and sensitivity to antiviral agents and antisera.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

David Derse — Senior Staff Fellow — LVC — NCI

Objectives:

1. Construct a recombinant bovine leukemia virus (BLV) in which the tax/rex genes are replaced with the neo gene controlled by the SV40 promoter.

2. Examine synthesis of viral proteins, production of infectious virus, and host range of cells susceptible to infection with the recombinant BLV.

3. Construct a recombinant human T-cell leukemia virus type I (HTLV-I) in which the tax, rex, and part of the env genes are replaced with the neo gene controlled by the cytomegalovirus (CMV) promoter.

4. Examine the pattern of viral proteins synthesized in cells, production of infectious virus, and host range of cells susceptible to infection with the recombinant HTLV-I.

Methods Employed:

The following techniques were employed: (1) recombinant plasmid construction, (2) transfections and infection of mammalian cells, and (3) Northern and Southern blot analysis.

Major Findings:

1. BLV-SVNEO is an activator-dependent retroviral vector. An intact provirus clone of BLV was deleted of the region between the end of the env gene and the 3′ long terminal repeat, thus removing the tax and rex genes without detriment to the structural protein genes. The bacterial neomycin resistance (neo) gene controlled by the SV40 early promoter was inserted into the 3′ end of the deleted BLV genome to produce BLV-SVNEO. Northern blot analysis of RNA isolated from cells transfected with plasmids containing BLV-SVNEO revealed that the neo gene was constitutively expressed at high levels. In contrast, BLV RNAs were produced from BLV-SVNEO only in those cells that supplied tax and rex in trans. These results suggested that infectious virus would be produced only when BLV-SVNEO was complemented with both tax and rex.

Cells were transfected with BLV-SVNEO in combination with tax and rex expression plasmids; the culture medium was collected two days later, filtered and added to a variety of cell cultures. The number of G418-resistant colonies that developed after exposure to the transfected-cell medium was proportional to virus titer. In the absence of tax or rex, BLV-SVNEO was not produced in the transfected cells. The human cell line, HeLa, was found to be highly susceptible to infection with BLV-SVNEO as judged by the large number of G418-resistant colonies that developed. Also sensitive to infection were

bovine, canine, feline and one of two murine cell lines (NIH 3T3 but not LTK- cells were infected). In contrast, two cell lines that are productively infected with BLV, FLK-BLV and BLV-bat cells, were resistant to superinfection with BLV-SVNEO, indicating that this is a receptor-mediated infection. Transfection of the FLK-BLV cell line with BLV-SVNEO followed by selection in G418 resulted in the establishment of a cell population that continuously releases high titers of BLV-SVNEO plus BLV. These cells will be useful as a source of BLV-SVNEO used in defining parameters that influence BLV infectivity.

2. Construction and expression of an HTLV-I recombinant virus. A similar strategy was applied to the development of an HTLV-I recombinant virus, designated HTLV-CMVNEO. An intact HTLV-I provirus clone was deleted of tax, rex, and part of the env gene; this region was replaced with the neo gene coupled to a CMV promoter. When transfected into cells, HTLV-CMVNEO directs the expression of neo at very high levels. Complementation with tax, rex, and env are essential for production of infectious virus. Experiments addressing the complementation, infectivity, and host range are in progress.

In summary, these activator-dependent retroviral vectors should facilitate molecular genetic studies of BLV and HTLV. These viruses have been very difficult to work with in the past due to their restricted and highly controlled gene expression. The neo viruses described here allow one to quantify virus infectivity independent of virus-controlled gene expression. These systems should help in defining specific receptors and in analyzing the effects of antiviral agents. Moreover, these studies demonstrate the feasibility of this approach in gene delivery to cells and perhaps to animals.

DEPARTMENT OF HEALTH AND HUMAN SERVICES · PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01CP05619-01 LVC

PERIOD COVERED

October 1, 1988 to September 30, 1989

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of Secondary Oncogenes in Plasmacytoma Acceleration by Avian v-myc

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Others:	Michael Potter	Chief	LG	NCI
	J. Frederic Mushinski	Senior Investigator	LG	NCI

COOPERATING UNITS *(if any)*

University Hospital, Innsbruck, Austria (J. Troppmair); Laboratory of Immunopathology, NIAID, Bethesda, MD (H.C. Morse)

LAB/BRANCH

Laboratory of Viral Carcinogenesis

SECTION

Viral Pathology Section

INSTITUTE AND LOCATION

NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
0.5	0.4	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Intraperitoneal injection of pristane in BALB/cAn mice induces plasmacytomas, which show c-myc-activating chromosome (6;15) or (12;15) translocations. Plasmacytoma development can be accelerated by inoculation with oncogene-carrying retroviruses, such as abl-MSV, in which case the accelerated tumors still carry the c-myc activating translocation. In contrast, inoculation with a particular v-myc-carrying retrovirus, J3, not only accelerates plasmacytomagenesis but also abrogates the c-myc translocation, which provided the first direct evidence for a role of the translocation activated myc in tumor development. However, other constructs of the same retrovirus vector (J5, J5D), which expressed different versions of tumorigenic avian v-myc, did not accelerate plasmacytomagenesis. We have analyzed the molecular basis for the differential activity of J3 in plasmacytoma acceleration and found that it is due to reactivation of the deletion-inactivated gag-v-raf gene present in J3 but not in the other v-myc-containing J viruses. These findings (i) confirm the abrogation of translocation-activated c-myc by exogenous v-myc; and (ii) demonstrate that exogenous v-myc, while necessary for abrogation, is insufficient for plasmacytoma acceleration, which additionally requires reactivation of v-raf in J3. We have also isolated variants of J5D, J5D*, by recovery of virus from a lymphoblastic lymphoma in a NSF/N mouse injected as a newborn with J5D virus. J5D* induced morphological transformation of fibroblasts in culture and accelerated plasmacytomagenesis in BALB/cAn mice.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Ulf R. Rapp	Chief, Viral Pathology Section	LVC	NCI
Michael Potter	Chief	LG	NCI
J. Frederic Mushinski	Senior Investigator	LG	NCI

Objectives:

Constitutive activation of c-myc by chromosomal translocation occurs in 95% of pristane-induced plasmacytomas in BALB/cAn mice, rat immunocytoma and Burkitt lymphoma in man. Direct evidence for a causative role of myc gene deregulation came from studies in this lab using the J series of recombinant retroviruses. Intraperitoneal injection of pristane-conditioned BALB/cAn mice with the J3 virus, which harbors an activated MH2/MC29 v-myc gene as well as a raf gene inactivated by a frameshift mutation not only accelerated plasmacytomagenesis but also replaced the need for chromosomal translocation. However, other viruses carrying MC29 v-myc (J5) or a MC29/MH2 v-myc hybrid gene (J5D) failed to accelerate plasmacytoma development, although all three viruses share the ability to transform pre-B and B cells in vivo in newborn NSF mice. The aim of this study is the analysis of the molecular basis for the differential activity of J3 in plasmacytoma induction.

Methods Employed:

Experimental strategy was based on the search for genetic alterations in the viruses, which may have occurred during in vivo passage. After virus recovery genomic DNA as well as RNA from virus-infected cells is analyzed using virus- and oncogene-specific probes. The virus is cloned and sequenced using standard recombinant DNA technology.

Major Findings:

1. Virus recovered from the ascites of mice with J3-accelerated plasmacytomas, J3*, differs from J3 by its ability to induce formation of raf-type foci on NIH 3T3 cells. In pristane-primed mice J3* rapidly accelerated plasmacytomagenesis.

2. Molecular cloning of J3* revealed the presence of two deletions, one of 370 base pairs (bp) located in gag and a second of 520 bp in the env region, which is located 3' of v-myc in J3. The effect of these deletions is (i) the expression of a 55 kd gag-raf fusion protein instead of the J3-specific 37 kd gag protein and (ii) a decrease in the ratio of v-myc subgenomic- over genomic-sized transcripts.

3. A variant of J5, J5D*, was recovered from a lymphoblastic lymphoma in a NSF/N mouse injected as a newborn with J5D virus. J5D* induces morphological transformation of fibroblasts in culture and accelerates plasmacytoma

development in BALB/cAn mice. Data obtained so far show no sign of involvement of a-raf.

Publications:

Troppmair J, Huleihel M, Cleveland J, Mushinski JF, Kurie J, Morse HC III, Wax JS, Potter M, Rapp UR. Plasmacytoma induction by J series of v-myc recombinant retroviruses: evidence for requirement of two (raf and myc) oncogenes for transformation. Curr Top Microbiol Immunol 1988;141:110-4.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01CP05620-01 LVC

PERIOD COVERED
October 1, 1988 to September 30, 1989

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Development of Vaccines and Antivirals Against Retrovirus Infection in Primates

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Raoul E. Benveniste	Medical Officer	LVC	NCI
Others:	Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

COOPERATING UNITS (if any) Bionetics Research, Inc., Frederick, MD (L. Henderson, P. Powell, A. Rein, R. Sowder); Oncogen, Seattle, WA (S.L. Hu); Univ. of Washington, Seattle, WA (W. Morton, C.-C. Tsai); Walter Reed Army Inst. of Research (D. Burke); USAMRIID, Frederick, MD (P.B. Jahrling)

LAB/BRANCH
Laboratory of Viral Carcinogenesis

SECTION
Immunogenetics Section

INSTITUTE AND LOCATION
NCI, NIH, Frederick, Maryland 21701-1013

TOTAL MAN-YEARS	PROFESSIONAL	OTHER
1.2	0.6	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A single-cell clone of HuT-78 cells infected with the simian immunodeficiency virus, SIV/Mne (clone E11S), produces virions that contain large amounts of gp120 envelope glycoprotein even after banding on sucrose gradients. Studies are underway to determine the in vivo infectious titers of stocks of this clone in macaques. Animals vaccinated with the psoralyn-inactivated clone, E11S, will have their neutralization titers determined prior to challenge with an appropriate titer of virus.

An invariant amino-sequence that can be found in all retroviruses is the cysteine array present in the nucleic acid binding protein. Recent studies with Moloney murine leukemia virus have shown that substitutions of cysteine residues for serine results in mature virus particles that lack any detectable viral RNA and are, therefore, noninfectious. Experiments are underway to perform site-directed mutagenesis in this region of the infectious molecular clone of SIV/Mne; if similar results are obtained, this altered clone will be useful in vaccination studies, since the absence of viral RNA will make recombination with endogenous retroviral sequences unlikely.

Simian AIDS (SAIDS) is an endemic disease of macaques that is etiologically linked to infection by a type D retrovirus. We have isolated and cloned a type D retrovirus (SRV-2/WASH) isolated from a macaque with retroperitoneal fibromatosis and SAIDS. A recombinant vaccinia virus (v-senv5) that expresses the envelope glycoprotein of SRV-2/WASH has been constructed and inoculated into macaques. Four v-senv5-immunized animals, together with four controls, were challenged intravenously with 10^3 infectious virus particles of SRV-2/WASH. Three of four controls became infected and one died at 7 weeks of SAIDS. In contrast, all four v-senv5-immunized animals remain healthy, virus-free, and seropositive only against the immunizing env-antigens 53 weeks later.

Project Description

Names, Titles, Laboratory and Institute Affiliations of Professional Personnel Engaged on this Project:

Raoul E. Benveniste	Medical Officer	LVC	NCI
Gisela Fanning-Heidecker	Staff Fellow	LVC	NCI

Objectives:

To develop vaccines to protect against retrovirus infection in nonhuman primates to serve as a model for retroviral vaccines in man.

Methods Employed:

Molecular cloning, sequencing, and site-directed mutagenesis of various Simian immunodeficiency viruses (SIV) in order to determine the molecular basis of pathogenicity. Isolation of single-cell clones of infected cells with unusual properties by using feeder layers of primary sheep choroid plexus cells in microtiter plates. Antibodies to viral proteins were detected by Western immunoblot techniques. Virus neutralization assays were performed by a rapid microtiter plate assay developed in this laboratory that measures appearance of syncytia after infection of cells by SIV or type D retroviruses.

Major Findings:

1. Vaccination of macaques with a single-cell clone of SIV/Mne that produces large amounts of envelope glycoprotein. Several single-cell clones of HuT-78 cells infected with end-point diluted SIV/Mne were obtained by selecting colonies that grew in microtiter plate wells seeded with a feeder layer of sheep choroid plexus cells. One of these, clone E11S, has large amounts of gp120 env glycoprotein associated with virus particles even after sucrose gradient purification. Studies are underway in collaboration with USAMRIID and Walter Reed Army Institute of Research to titer a stock of E11S in vivo in macaques. This stock has an in vitro titer of 1-4.9 x 10^5 virus particles/ml. Animals (two per dilution) are being inoculated intravenously with 10^4 to 10^{-2} in vitro doses of virus. A separate group of macaques will be vaccinated with the psoralyn-inactivated clone, E11S, the neutralization titers determined, and challenged with an appropriate dose of E11S based on the previously determined in vivo titer. Since clone E11S has large amounts of native gp120, these experiments will determine if protection can be elicited.

2. Site-directed mutagenesis of the cysteine array of an SIV/Mne molecular clone. All retroviruses contain a small basic gag protein that binds to single-stranded nucleic acids and contains one or two copies of the following invariant structure: -Cys-R-R′-Cys-X-X-X-Gly-His-X-X-X-X-Cys-, where either R or R′ is a residue with an aromatic side chain (Phe, Tyr, Trp, or His) and X represents variable residues. Site-directed mutagenesis of Cys to Ser of Moloney murine leukemia virus resulted in virions with a normal morphology that lacked any detectable viral RNA and were noninfectious. We are performing similar experiments on a molecular clone of SIV/Mne. If these

mutants also lack viral RNA, they will be potentially valuable immunogens since there will be no possibility of recombination with endogenous viral sequences.

3. Protection of macaques against simian AIDS by immunization with a recombinant vaccinia virus expressing the envelope glycoproteins of simian type D virus. Simian AIDS (SAIDS) is an endemic disease of macaques that shares many characteristics with AIDS in humans. SAIDS is etiologically linked to infection by a type D retrovirus (SAIDS retrovirus, or SRV). Immunization with inactivated whole virus vaccine has been shown to protect macaques against SRV infection. To identify the antigen(s) responsible for eliciting protective immunity, we have constructed a recombinant vaccinia virus (v-senv5) that expressed the envelope glycoproteins of a molecular clone of SRV serotype 2 (SAIDS-D/WASH), the type D virus associated with simian AIDS and retroperitoneal fibromatosis at the University of Washington Primate Center. Macaques immunized with v-senv5 showed lymphoproliferative responses to purified SRV and generated antibodies that neutralized SRV-2/WASH infectivity in vitro and mediated antibody-dependent cellular cytotoxicity against SRV-2-infected cells. Four v-senv5-immunized animals, together with four control animals, were challenged intravenously with 1 x 10^3 tissue culture ID_{50} of SRV-2. As early as 2 weeks post-challenge, three of four control animals became viremic, and two of these three animals also seroconverted. The animal that was viremic, but remained antibody negative, died of symptoms of SRV-infection at 6 1/2 weeks post-challenge. In contrast, all four v-senv5-immunized animals remain healthy, virus-free, and seropositive only against the immunizing env antigens 53 weeks after challenge. These results indicate that immunization with a recombinant vaccinia virus expressing the envelope antigens of SRV-2/WASH protects primates from a retrovirus-induced immunodeficiency disease.

Publications:

Shiu-Lok H, Zarling JM, Chinn J, Travis BM, Moran PA, Sias J, Kuller L, Morton WR, Fanning-Heidecker G, Benveniste RE. Protection of macaques against simian AIDS by immunization with a recombinant vaccinia virus expressing the envelope glycoproteins of simian type D virus. Proc Natl Acad Sci USA (In press).

Tsai C-C, Follis KE, Benveniste RE. Antiviral effects of 3′-azido-3′-deoxythymidine, 2′,3′-dideoxycytidine, and 2′,3′-dideoxyadenosine against simian acquired immunodeficiency syndrome-associated type D virus (SAIDS-D/WA) in vitro. AIDS Res Hum Retroviruses 1988;4:359-68.

ANNUAL REPORT OF

BIOLOGICAL CARCINOGENESIS BRANCH
BIOLOGICAL CARCINOGENESIS PROGRAM
DIVISION OF CANCER ETIOLOGY
NATIONAL CANCER INSTITUTE

October 1, 1988 to September 30, 1989

The Biological Carcinogenesis Branch (BCB) plans, develops, directs and manages a national extramural program of basic and applied research concerned with the role of biological agents as possible etiological factors or co-factors in cancer and on the control of these agents and their diseases; establishes program priorities, and evaluates program effectiveness; provides a broad spectrum of information, advice and consultation to individual scientists and institutional science management officials relative to NIH and NCI funding and scientific review policies and procedures, preparation of grant applications and choice of funding instruments; provides NCI management with recommendations as to funding needs, priorities and strategies for the support of relevant research areas consistent with the current state of development of individual research activities and the promise of new initiatives; plans, develops and manages research resources necessary for the conduct of the coordinated research program; develops and maintains computerized data management systems; plans, organizes and conducts meetings and workshops to further the program objectives; and maintains contact with the relevant scientific community to identify and evaluate new research trends relating to its program responsibilities.

The objectives of the research program are accomplished through a variety of extramural support mechanisms including traditional individual research project grants (R01), conference grants (R13), academic research enhancement (AREA) awards (R15), first independent research support and transition (FIRST) awards (R29), outstanding investigator grant (OIG) awards (R35), the method to extend research in time (MERIT) awards (R37), program project grants (P01), cooperative agreements (U01), contracts (N01), and small business innovative research (SBIR) grants and contracts (R43/44, N43/44). Currently, the Branch administers 409 research grants with an annual budget of approximately 83 million dollars. Administratively, the Branch is divided into seven functional entities which are shown in Figure I. The Branch scientific components are based primarily on the major subdivision of viruses by their type of nucleic acid core. Research programs on viruses with a DNA core which are involved in the induction of malignant transformation are included in the DNA Virus Studies components. The component designated DNA I deals with research on the two main groups of large DNA viruses, the herpesviruses and adenoviruses. The DNA II component supports research on the small DNA viruses, the polyoma, simian virus 40 (SV40), and papillomaviruses. Similarly, research dealing with RNA core viruses are covered by the RNA Virus Studies components. The component designated RNA I involves research concerning murine, feline, bovine, nonhuman primate, and human viruses. The RNA II component incorporates research involving avian tumor viruses, picornaviruses, hepatitis B virus, and other microbial agents. The Research Resources component arranges for the storage and distribution of research materials, helps

oversee the various resource contracts, and maintains computerized information systems covering the distribution of resources. The AIDS Virus Studies component was established to deal with the increasing concern about this public health problem. Originally, acquired immunodeficiency syndrome (AIDS) activities in the program were treated as special initiatives administered by the Branch Office. However, the high visibility of these efforts and the need for an accountable individual to expedite reports and responses to senior echelons dictated that a program director be assigned exclusively to these research oversight activities. Finally, the Office of the Branch Chief oversees and coordinates all of these activities, establishes program objectives and priorities, evaluates accomplishments, and interacts with the Division and Institute leadership.

The research resources payback system of the Branch has been described in previous reports. During this period, all four traditional resource contracts were functioning in the payback mode. These include one for production of viral reagents, one for specialized testing services, one for supplying animals, and one for storage and distribution of frozen biological reagents. The payback system seems to be performing as expected. The demand for high quality biological reagents, not readily available from commercial sources, has remained fairly constant. Reimbursement for full or partial costs of services has led to more careful use by investigators of costly resource reagents, with a subsequent reduced level of effort in several resource contracts or the termination of now unnecessary activities.

Table I focuses on mechanisms of support of extramural research and related activities in biological carcinogenesis. The total BCB grant and contract budget in FY89 is estimated to be about 84 million dollars. It should be noted that the Branch now administers 22 program project grants at a level of 20.26 million dollars, 18 outstanding investigator awards at the level of 14.4 million dollars, and 25 merit awards at a level of 4.9 million dollars. Table II provides an estimate of the grant and contract support, respectively, in each of the Branch components and thus illustrates, in quantitative terms, the main areas of scientific endeavor. As can be seen from the table, the four primary long-established Branch research components are well balanced in terms of their number of grants and funding level. The AIDS component, while smaller in size, is now also a fully functioning entity. The Branch currently administers 409 grants and 13 contracts. Table III summarizes the specific targeted research activities initiated by the Biological Carcinogenesis Branch since 1982. The table demonstrates the broad spectrum of research activities, funded through the traditional grant and cooperative agreement mechanisms, undertaken to stimulate activity in newly emerging areas of scientific opportunity.

During FY89, the Branch was again active in sponsoring a variety of research initiatives which reflected emerging areas of research opportunity. Traditional research projects were funded that resulted from two requests for grant applications (RFAs), one new RFA was issued, and three Branch-sponsored workshops were held during the fiscal year.

As a result of previous Branch workshops held in 1987, two RFAs had received concept approval by the Division of Cancer Etiology (DCE) Board of Scientific Counselors (BSC) during FY88 and were funded during this fiscal year. The first was entitled "Animal Models for Human Papillomavirus-Associated Neoplastic Diseases" and was based on a workshop which met in September 1987. The sum of $725,000 was approved to stimulate basic research to define the host response

underlying the frequently observed regression of papillomavirus lesions which occur in man and animals. A secondary goal was the promotion of studies on the mechanism(s) of progression of dysplastic papillomavirus-associated lesions to frank carcinoma. The second initiative, entitled "Retrovirus Animal Models and HIV Pathogenesis" was based on a workshop held in October 1987. The sum of $1,000,000 was initially approved to fund studies on human immunodeficiency virus (HIV) pathogenesis and on virus-host interactions in order to better define and understand HIV-induced immune dysfunctions. Because of the number of high quality applications addressing a broad spectrum of animal models, 1.3 million dollars was eventually obligated. Thus, a total of nine new traditional research projects were funded as a result of the responses received to these two RFAs.

Additionally, a Branch workshop held in April 1988, on "Mechanisms of Pathogenic Diversity of Epstein-Barr Virus" was co-sponsored with the National Institute of Allergy and Infectious Diseases (NIAID). The workshop participants identified a number of areas of Epstein-Barr virus (EBV) research in which there are gaps in our knowledge. These gaps result primarily from the lack of a lytic replicative system for EBV which would allow investigators to readily study the virus in vitro. The Branch developed a concept which, after DCE BSC approval in FY89, resulted in an RFA entitled "New Approaches to Studying Epstein-Barr Virus Oncogenesis." The goals of this RFA are to develop and apply new molecular biological methods, such as recombinant DNA technology, to enable investigators to study EBV gene functions and thereby start to really understand the mechanisms of oncogenesis by this virus. The sum of $850,000 was approved by the Board to fund meritorious applications in response to this RFA. The RFA was issued in January 1989, with a receipt date of August 3, 1989, and anticipated funding in FY90.

The Office of the Branch Chief and the RNA Virus Studies II component were instrumental in planning an "International Workshop on Pathogenesis and Prevention of Hepatocellular Carcinoma," held in February 1989, which was co-sponsored by the Fogarty International Center and the National Cancer Institute, in cooperation with the Cancer Institute of the Chinese Academy of Medical Sciences and Merck & Company. During that meeting, the importance of primary hepatocellular carcinoma (PHC) as a major worldwide human malignancy was stressed (over 1,000,000 deaths per year worldwide). The additional information that over 300,000,000 chronic hepatitis B virus (HBV) carriers worldwide will die of liver cancer in the absence of some prevention or intervention strategies, furnished additional impetus for studies on etiologic mechanisms, intervention strategies, and prevention activities directed against this fatal human malignant disease. It was clear from the discussions at the workshop that the mechanisms of hepato-carcinogenesis, whether virally induced, chemically induced, or requiring both factors, remain a matter of conjecture and require additional studies for elucidation. There are no currently funded NCI grants investigating such topics as HBV variants, the possible role of non-A/non-B hepatitis in hepatomas, or systematically studying the cancer risks of those individuals who are refractory to vaccination. In view of these considerations, the RNA II component plans to seek approval from the DCE Board of Scientific Counselors for the issuance of an RFA in FY91, setting aside targeted NCI monies to fund meritorious applications dealing with these aspects of liver cancer.

In April 1989, the DNA Virus Studies II component sponsored a workshop entitled "Interactions of T-Antigens with Proto-Oncogenes." The participants at the meeting agreed that several areas of research might benefit from specific

stimulation via the grant or contract mechanism. These suggestions are currently undergoing staff review. In May 1989, the RNA Virus Studies I component sponsored a workshop entitled "Retroviruses and Human Disease: Search for New Agents." The attendees at this workshop also suggested a number of potentially fruitful areas for research which are currently undergoing staff evaluation and review.

Research sponsored by the Branch has yielded a variety of fundamental information on biological carcinogenesis by studying human and animal tumor viruses which induce cells to become malignant. These viruses provide tools for understanding how cellular genes and their products convert a normal cell into a tumor cell. Of equal importance, horizontally transmitted particulate human viruses may, themselves, be directly responsible for some malignant transformations in man. Investigations of biological carcinogenesis have shown that viral information present as nucleic acid sequences in the genes of normal cells and replicating with them may be intimately involved in the development of cancer. This may occur either through the direct effects of viral transforming genes; through the influence of viral enhancing elements (promoters or long terminal repeats) on cellular oncogenes; or through the interaction of viruses with a variety of environmental factors, such as chemicals, radiation, and the like. In the research program, attention is given to studies defining the interaction of viruses and cells in both animal and human cancers.

Recent studies have shown that a number of rapidly transforming RNA viruses have derived their ability to cause cancer in animals, and perhaps in humans, from genes found in normal cells. These genetic sequences are unrelated to those necessary for normal viral replication and have been found to produce proteins which are, in many cases, necessary for the initiation of the transformed state. Since the gene products result in transformation, the genes have been termed "oncogenes." Oncogenes have been found to possess the following general characteristics: they are essential for certain types of transformation; they are derived from cellular genetic sequences; they have a limited number of specific targets; they act by means of their translational protein products; and they are probably limited in number (approximately 40 have been found). Currently, ongoing research seeks to continue the search for oncogenes in both animal and human tumor systems and to characterize these genes; to study human oncogenes and their relationship(s) to viral oncogenes in terms of nucleic acid homology and translational (oncogene) products; to purify and characterize the translational gene products of these genes; to use these purified products in delineating the mechanism(s) of transformation; and to define the function(s) and mechanisms of regulation of the cellular homologs (c-oncs) of viral transforming genes. Research highlights of the past year are presented here and, in greater detail, in the various section reports which follow.

A class of recessive oncogenes, called anti-oncogenes or suppressor oncogenes, has been discovered in which the absence of the oncogene product, rather than its presence, is responsible for transformation. One of the best understood examples of suppressor genes is the Rb gene of retinoblastoma, which appears to play an important role in the induction of retinoblastoma, as well as other commonly occurring cancers, such as cancers of the lung and colon. Loss or inactivation of both copies of the Rb gene in humans may predispose an individual to cancer. It was recently found that loss of both copies of the Rb gene in retinoblastoma tumors results in loss of cellular response to the growth inhibitory substance, transforming growth factor beta (TGF-beta). Subsequently, it was demonstrated

that the mechanism for this loss of response by the tumor cells appears to result from loss of the cellular receptors for TGF-beta. These observations may explain the transformed phenotype of retinoblastoma cells.

A second observation suggests that inactivation of the Rb protein may be a mechanism of oncogenesis used by as many as three groups of DNA viruses. Adenoviruses, papillomaviruses, and polyomaviruses all encode specific viral proteins responsible for the malignant transformation of infected cells. Some of these same viral transforming proteins have been demonstrated to bind tightly to the retinoblastoma protein. Since cancer is promoted by the loss or inactivation of the Rb gene, and consequently of the Rb protein, the simplest hypothesis for oncogenesis by these viruses is the inactivation of the Rb protein as a result of complex formation with viral transforming proteins.

A presumptive second anti-oncogene has been identified in studies using the small DNA tumor virus SV40. The cellular protein product of this anti-oncogene, designated p53, was initially recognized by its ability to bind the transforming protein of the SV40 large T-antigen. p53 was at first thought to be an oncogene, since it appeared to cooperate with the ras oncogene to transform primary cells in culture. Recent studies have demonstrated that the p53 DNA used in those studies represented mutant p53 which had lost its ability to act as an anti-oncogene. Experiments with unmutated normal p53 demonstrated its ability to inhibit transformation by two genes that would in combination normally transform cells (the adenovirus E1A gene and ras oncogene). One way in which SV40 T-antigen may transform cells is by binding to and inactivating the p53 protein. The study of the cellular proteins to which viral transforming genes bind may provide a useful approach to identifying additional cellular anti-oncogenes.

Adenoviruses are a group of medium sized DNA viruses whose oncogenicity has been demonstrated by their ability to cause in vitro transformation of epithelial cells and in vivo tumors in newborn rodents. Extensive analysis of the adeno-virus DNA sequences has demonstrated that the E1A and E1B subregions of the E1 gene contain the genetic information necessary for transformation. Recent studies have demonstrated that another region of the viral genome, the E3 gene, plays a key role in transformation. This early region encodes at least seven messenger RNAs (mRNAs), three of whose proteins are involved in altering cell properties which affect the host's response to virus-transformed cells. The first protein, gp19, binds to class I major histocompatibility complex (MHC) antigens and prevents MHC antigen transport to the cell membrane. Thus, the gp19 protects transformed cells from lysis by cytotoxic T-cells. Synthesis of the second, 14.7 kD protein, has been shown to protect cells from cytolysis by tumor necrosis factor. Finally, the 10.4 kD protein appears to down-regulate the synthesis of epidermal growth factor in the early stages of adenovirus infection. This change in the host cell may alter the availability of cellular factors needed for adenovirus gene transcription. Thus, the in vivo functions of the E3 gene appear to be highly relevant to the process of adenovirus transformation.

A novel approach to studying oncogene function involves production of mutants of a v-src-transformed rat cell line which are no longer phenotypically transformed despite the continued expression of wild-type alleles of v-src. This cell line appears to be resistant to re-transformation by several oncogenes in addition to v-src. The responsible mutation seems to be a dominant cellular gene. Further studies of this cell line are ongoing, and attempts to isolate the mutant host

gene by retroviral insertion mutagenesis could provide interesting clues to the mechanism of this resistance to transformation.

Significant observations have also been made in studies of another group of retroviruses, the human immunodeficiency viruses (HIV). The discovery of proteins unique to HIV-1 and HIV-2 may provide serological reagents to assess the prevalence of each type of HIV in different population groups. An open reading frame, termed orf-u, was discovered within the HIV-1 genome with the potential to encode a viral protein of about 80 amino acids. Since the orf-u region is not found in HIV-2, antibody to the protein product of this region may serve as a marker to distinguish between HIV-1 and HIV-2 infections. Another open reading frame, orf-x, has been demonstrated in HIV-2 and SIV (simian immunodeficiency virus), but not in HIV-1. While the functions of the HIV-1 orf-u and HIV-2 orf-x proteins are unknown, these proteins or antibodies to them should be useful in allowing researchers and clinicians to distinguish between HIV-1 and HIV-2 infections.

The CD4 molecule on T-lymphocytes is the receptor for the HIV-1 and SIV viruses. Recent in vitro studies have demonstrated that the recombinant soluble form of CD4 (rsCD4) is a potent inhibitor of replication of both viruses. SIV-infected rhesus monkeys, which have a disease similar to human AIDS, received daily intramuscular injections of rsCD4 to assess the therapeutic efficacy of rsCD4 in preventing SIV infection or its associated immunodeficiency disease. Isolation of SIV from peripheral blood lymphocytes became increasingly difficult as therapy continued. However, between 60-90 days after the termination of therapy, virus could again be isolated from the animals. The rsCD4 may act by several mechanisms: by absorbing soluble SIV envelope glycoprotein and thus diverting cytolytic T-cells from killing infected lymphocytes; by inhibiting the fusion of virus-infected cells with uninfected cells, thus, inhibiting the spread of virus; or by directly blocking the interaction of virus with CD4 molecules on target T-cells. Thus, this material could have potential value in the treatment of AIDS patients.

HTLV-1 and HTLV-2 viruses cause various forms of T-cell leukemia and lymphoproliferative disorders, and can immortalize peripheral blood T-cells in vitro. Since some AIDS patients are also infected with HTLV-1 or HTLV-2, studies were undertaken to determine whether HTLV augments HIV production. After mitogenic stimulation by noninfectious HTLV-1 virions, peripheral blood leukocytes infected in vitro with HIV-1 produced large quantities of HIV-1. The HTLV-1 virions exerted this effect prior to, immediately following, or well after the cells were infected with HIV-1. These results provide further impetus for studies of dually infected individuals to determine whether HTLV-1 may act as a cofactor in clinical AIDS. It is anticipated that future studies will determine whether HTLV-2 can exert a similar effect.

In studies of animal models of human liver cancer, hepatocellular carcinomas induced in two woodchucks chronically infected with the woodchuck hepatitis virus were characterized for viral integration near c-myc and for alterations of c-myc expression. Amplification of c-myc has been reported in cases from a wide variety of neoplasms, including leukemias and carcinomas. In both cases, insertion of the viral enhancer and disruption of normal c-myc transcriptional or post-transcriptional control appeared to be involved in c-myc activation. Integration of woodchuck hepatitis virus near a cellular proto-oncogene appears to have a role in the etiology of liver tumors.

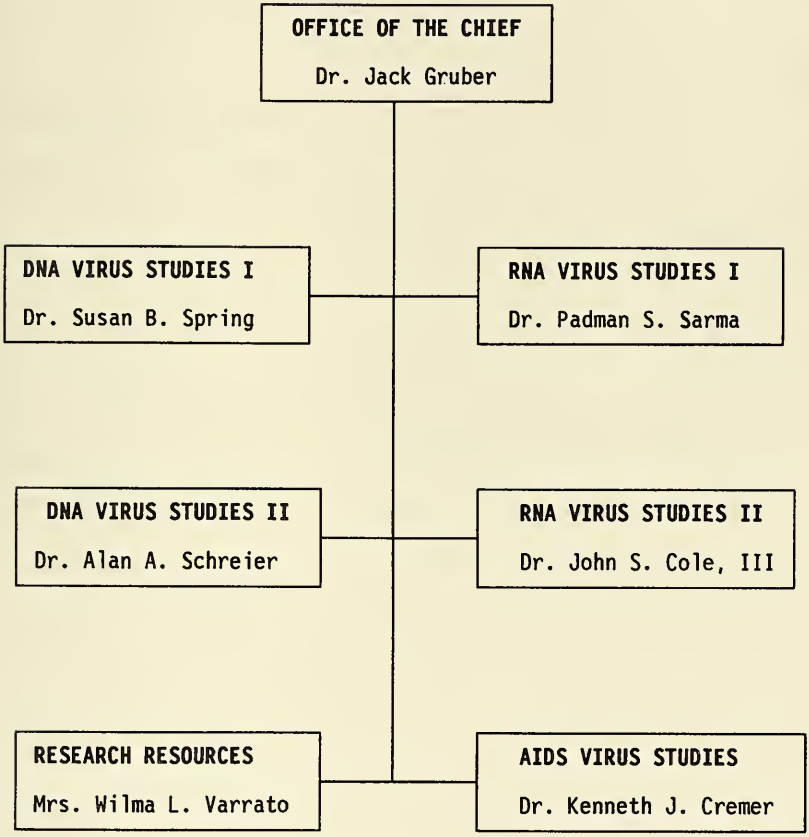
Two vectors containing viral DNA sequences have been developed which may prove useful for studies of virus oncogenesis and for gene therapy, respectively. A retrovirus vector carrying an early adenovirus gene can be used to immortalize epithelial cells. Although most human neoplasias are derived from epithelial cells, investigators have often been limited to studying fibroblast transformation because of difficulties of propagating primary epithelial cells in vitro. Thus, this new vector may facilitate studies of epithelial cell transformation by providing a source of the cells. The second plasmid contains the DNA sequences of the two viral origins of replication of Epstein-Barr virus DNA and the terminal sequences of the viral genome. It is anticipated that genes of therapeutic value could be added to this plasmid and that the encapsidated pseudovirus formed from it would target host B-cells. This vector would be non-lytic in such cells and thus allow the expression of the therapeutic gene.

Studies of the Rous sarcoma virus gag gene have provided a novel method for the synthesis of proteins in mammalian cells. A major difficulty in producing proteins on a large scale in mammalian cells has been the contamination of the desired protein by large amounts of serum proteins used in the growth medium. Retroviral vectors have been produced in which the gag gene is linked to the unrelated protein of interest. This technique results in the production of fusion proteins which migrate to the cell surface. Interaction of the gag portion of the molecules with one another results in budding, in which the fusion proteins become membrane enclosed and other cytoplasmic proteins are excluded. As the budding process nears completion, the protein of interest is cleaved from the retrovirus protein by a cellular protease associated with the site of budding. The enveloped particles are then released into the growth medium and can be purified by centrifugation in the same way that a retrovirus can be purified. Since only one of the retrovirus genes is used in the process, the particles are not infectious, are safe to handle, and offer a convenient means of large-scale protein production in mammalian cell systems. This technique, called "retro-secretion," is currently being patented by the grantee institution.

Studies on the DNA tumor virus SV40 have demonstrated a possible mechanism for the host immune system to detect and respond to viral antigens which are usually sequestered due to their nuclear location. Cytotoxic T-lymphocyte (CTL) clones have been isolated which recognize and lyse SV40-transformed cells. This recognition requires presentation of the processed T-antigen sequences on the surface of transformed cells by a particular antigen of the MHC (major histocompatibility complex). Single amino acid changes in this MHC antigen can abolish recognition of some of the T-antigen epitopes by the CTLs. Such allelic differences in MHC antigens could represent a mechanism for genetic susceptibility to viral oncogenesis.

Thus, the BCB has supported a variety of studies on both RNA and DNA viruses. These studies have demonstrated novel mechanisms by which some of these agents cause oncogenic transformation and/or cancer. In addition, a number of new scientific initiatives have been developed. Although the seminal questions of how viral oncogenes transform cells and how cellular oncogenes may be related to human cancer have yet to be answered, the research activities carried out by the BCB are providing the fundamental information necessary for their ultimate resolution.

FIGURE I


OFFICE OF THE CHIEF
Dr. Jack Gruber
DNA VIRUS STUDIES I
Dr. Susan B. Spring
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DNA VIRUS STUDIES II
Dr. Alan A. Schreier
RNA VIRUS STUDIES II
Dr. John S. Cole, III
RESEARCH RESOURCES
Mrs. Wilma L. Varrato
AIDS VIRUS STUDIES
Dr. Kenneth J. Cremer

TABLE I

BIOLOGICAL CARCINOGENESIS BRANCH
EXTRAMURAL PROJECTS ACTIVE FY 1989
(dollars in thousands)
(estimated)

	GRANTS/CO-OPS/CONTRACTS	
	NUMBER	DOLLARS
Research Grants		
Traditional Project Grants (R01)	296	40,397
Conference Grants (R13)	15	32
Academic Research Enhancement Awards (R15)	1	0
First Independent Research Support and Transition (FIRST) Awards (R29)	29	2,670
Outstanding Investigator Grants (R35)	18	14,427
Method to Extend Research in Time (MERIT) Awards (R37)	25	4,941
Program Project Grants (P01)	22	20,260
Cooperative Agreements (U01)	3	0
SBIR Research Contracts (N43/44)	9	561
Research Resources Contracts (N01)	4	659
TOTAL	422	83,947

TABLE II

BIOLOGICAL CARCINOGENESIS BRANCH

Contracts and Grants Active During FY 1989

	FY 89 (Estimated)			
	CONTRACTS		GRANTS	
	No. of Contracts	$ (Millions)	No. of Grants	$ (Millions)
DNA Virus Studies I	4	0.21	86	18.29
DNA Virus Studies II	3	0.25	108	21.11
RNA Virus Studies I	-	-	100	17.12
RNA Virus Studies II	-	-	91	21.35
AIDS Virus Studies	2	0.10	24	4.86
Research Resources	4	0.66	-	-
TOTAL	13	1.22	409	82.73

TABLE III

BIOLOGICAL CARCINOGENESIS BRANCH
Research Initiatives 1982-89

Title	Date Workshop	Date BSC Review	AWARDS 1ST YEAR FY	No.	Total Dollars
NIH-NCI-DCT-CTRP-82-13 (COOP) Studies of Acquired Immune-Deficiency Syndrome (KS & Opportunistic Infections)	-	May 82	83	5	$ 962,575
NIH-NCI-DCCP-82-18 (RFA) Hepatitis B Virus and Primary Hepatocellular Carcinoma	May 82	Sep 82	84	8	$1,073,037
NIH-NCI-DCCP-BCB-83-3 (COOP) Infectious Etiology of AIDS and Kaposi's Sarcoma	-	Feb 83	84	11	$1,537,613
NIH-NCI-DCE-BCB-84-19 (COOP) Studies on Bovine Leukemia	May 83	Mar 84	85	4	$ 380,758
NIH-NCI-DCE-BCB-84-27 (COOP) Studies on Human T-cell Leukemia & Lymphoma Virus Types I & II	Apr 84	Jun 84	85	7	$ 690,272
NIH-NCI-DCE-85-10 (RFA) The Role of Human Papillo-mavirus in the Etiology of Cervical Cancer	Jun 84	Oct 84	86	7	$ 763,074
NIH-NCI-DCE-85-20 (RFA) Basic Studies on the Development and Assessment of Retroviral Vaccines	Dec 84	Feb 85	86	4	$ 594,667
NIH-NCI-DCE-85-21 (RFA) Studies on Novel Human Exogenous and Endogenous Retroviruses	Mar 85	May 85	86	4	$ 547,709

TABLE III (cont.)

BIOLOGICAL CARCINOGENESIS BRANCH
Research Initiatives 1982-89

Title	Date Workshop	Date BSC Review	AWARDS 1ST YEAR--- FY	No.	Total Dollars
NIH-NCI-DCE-86-07 (RFA) The Transformation Mechanisms of Human Polyomaviruses	Mar 85	Oct 85	87	6	$ 771,480
NIH-NCI-DCE-87-19 (RFA) Studies on Papillomavirus-Host Interactions	Feb 86	Oct 86	88	5	$ 777,796
NIH-NCI-DCE-87-18 (RFA) Studies of Functional Anti-Sense RNA in Oncogenic Viral Systems	Mar 86	Oct 86	88	3	$ 346,210
NIH-NCI-DCE-88-13 (RFA) Animal Models for Human Papillomavirus-Associated Neoplastic Diseases	Sep 87	Feb 88	89	2	$ 390,031*
NIH-NCI-DCE-88-14 (RFA) Retrovirus Animal Models and HIV Pathogenesis	Oct 87	Feb 88	89	7	$1,294,066*
NIH-NCI-DCE-89-08 (RFA) New Approaches to Studying EBV Oncogenesis	Apr 88	Oct 88	90		$ 850,000**

* Estimated Funding
** Authorized Funding

SUMMARY REPORT

DNA VIRUS STUDIES I

The DNA Virus Studies I component of the Branch involves research on two groups of large DNA viruses, the herpes- and adenoviruses. In this component, extramural research is supported primarily by the grant mechanism. There are 86 research grants with an estimated total funding level of 18.29 million dollars. These include the traditional research grants, program project grants, conference grants, first independent research support and transition (FIRST) awards, method to extend research in time (MERIT) awards and outstanding investigator grants. The major research emphasis lies in studies of the mechanism(s) of viral transformation, which include genome structure, gene function and expression (68%); and virus-cell interaction (32%). In terms of the viruses being studied, 26% involve herpes simplex virus (HSV), 24% involve Epstein-Barr virus (EBV), 6% involve cytomegalovirus (CMV), 12% involve other herpesviruses, and 31% involve adenoviruses. In addition, the component supports four Phase I small business innovative research (SBIR) contracts aimed at developing monoclonal antibodies and molecular probes for oncogenic herpesviruses.

Investigators supported by this program are attempting to elucidate the mechanism(s) of transformation of herpes- and adenoviruses by a variety of approaches, including localization of transformation function(s) to specific sequences of the viral genome, elucidation of the function(s) of individual viral genes, and determination of the mechanism(s) of regulation of synthesis of viral gene products. Studies with a more biological orientation have investigated virus-host interactions in order to define the process of viral pathogenesis.

Herpesviruses

Most members of the herpesvirus family can transform cells in vitro and all of them can establish latent infections in man and animals. Many of the herpesviruses have been suspected of having a role in tumor induction in man, either directly or as cofactors. Because all herpesvirus infections result in life-long latent infections, reactivation of these viruses during immunosuppressive therapies is a cause of morbidity and mortality among cancer patients and transplant recipients.

Epstein-Barr virus is a lymphotropic herpesvirus which has been associated with several disease entities including infectious mononucleosis, Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). More recent evidence suggests an etiological role for EBV in B-cell lymphomas in immunocompromised individuals and in oropharyngeal tumors. When EBV infects epithelial cells in vivo, the virus undergoes a complete replicative cycle, thus providing infectious virus for the transmission of EBV infection from one individual to another. In contrast, when EBV infects B-lymphocytes in vivo or in vitro, the infection is primarily latent. However, these B-cells are altered, and unlike normal lymphocytes, B-lymphocytes latently infected with EBV can proliferate indefinitely and can be cloned and grown as continuous cell lines. Despite the presence of the complete virus genome, only a few virus genes are expressed. Because of the difficulties involved in obtaining epithelial cells for in vitro study, these latently

infected transformed B-cells have provided most of our knowledge of the molecular biology of EBV.

To understand the pathogenesis of EBV and its ability to induce both infectious diseases and neoplasia affecting two types of tissues, B-cells and epithelial cells, the viral and host factors responsible for these differences in disease state are being investigated. Researchers have identified a virus encoded transactivator, the Z transactivator, which plays a role in the transition from latent to replicative EBV infection. They have also identified a region in the virus genome, the Bam HI-I promoter, which is responsive to the Z transactivator. Studies of these regulatory sequences in the Bam HI-I promoter have demonstrated that there are at least two such elements, one of which confers responsiveness in lymphoid cells, while the other confers responsiveness in Vero (epithelial) cells. Thus, one of the factors which determines the nature of virus replication in different cell types has been ascertained (23).

Proto-oncogene activation may be another factor which regulates virus function in different cell types. Burkitt's lymphoma cells demonstrate characteristic chromosomal rearrangements which are associated with activation of the c-myc oncogene. When cell lines established from sarcomas, lymphomas and carcinomas were examined for the expression of another proto-oncogene, c-fgr, those cell lines which expressed elevated c-fgr were found to be infected with EBV, whereas EBV-negative tumor cell lines lacked c-fgr messenger RNA (mRNA). This observation suggested that EBV infection resulted in transcriptional activation of the c-fgr oncogene. The expression of c-fgr was also elevated in EBV-infected lymphoma tissues and cell lines established from lymphomas. However, c-fgr was not elevated in nasopharyngeal carcinoma tissue. These observations suggest that some differences in EBV pathogenesis may be attributable to differences in activation of the c-fgr oncogene (53).

While the site of EBV latency in man has not yet been determined, it has been proposed that there are either reservoirs of latently infected cells both in B-lymphocytes and in oropharyngeal cells or, alternatively, that B-lymphocytes are continuously infected from a reservoir in the oropharynx. Studies of recipients of allogeneic bone marrow transplants suggest that the reservoir for EBV infection is in the hematopoietic tissue. These studies are based on the ability to distinguish between EBV isolates by the differential migration of viral polypeptides on polyacrylamide gels. Using this technique, the phenotype of the EBV strain present in bone marrow transplant recipients before and after transplantation was studied. In one case, an EBV seropositive recipient had EBV virus with the phenotype of the bone marrow donor and completely lost the EBV virus that he had prior to receiving the transplant. In another case, an EBV seropositive recipient became EBV negative after receiving a transplant from an EBV negative donor. When this second patient became EBV positive 1200 days after transplantation, the EBV phenotype was different from her pretransplant phenotype. On the basis of these observations, it appears that latently infected EBV host cells reside in a cellular compartment that can be destroyed by the irradiation, cytotoxic drugs and graft-versus-host reactions that are part of the transplantation process. Thus, the most likely site for such cells would be the hematopoietic host tissues. If this is true, it may be possible to eradicate EBV infection by physical or chemical procedures (34).

In latently infected B-lymphocytes, EBV DNA is maintained as a circularized plasmid. The virus needs two types of mechanisms to duplicate its genome. One

type is involved in duplication of the virus genome when latently infected B-lymphocytes divide. The second type is used during the replicative cycle, when the virus makes multiple copies of its genome for insertion into new virions. Investigators have previously identified the origin of replication for the latent DNA plasmids and designated it ori-P. Ori-P is not capable of replicating viral DNA when lytic infection is induced by chemicals or by transactivation with viral genes (such as the Z transactivator discussed above). Recently, a second origin of EBV replication has been identified and designated as ori-Lyt. Comparison of the structure of this origin with that of other viral origins of replication suggest that some properties are common to several viruses, whereas others are unique to EBV. The structure of ori-Lyt consists of a minimal region required for function and auxiliary sequences that enhance function. This type of structure has analogies with the origins of replication of small and medium sized DNA viruses, such as papovavirus and adenoviruses. The presence of potential binding sites for transcription factors distinguishes ori-Lyt from the origins of herpes simplex virus types 1 and 2 (HSV-1 and HSV-2). It has been possible to construct plasmids containing three groups of EBV DNA sequences: ori-P, ori-Lyt and terminal regions. Plasmids of HSV-2 DNA having the corresponding sequence elements are capable of being packaged into virion-like structures. Thus, using this technology, it may be possible to construct a non-cytocidal EBV-vector targeted to human B-lymphocytes that could be used for therapeutic applications (RNA II component, reference 81).

The limited expression of EBV genes during latent infection is hypothesized to be a mechanism used by the virus to prevent the destruction of latently infected B-cells by the host cytotoxic T-cells. It has been demonstrated that Burkitt's lymphoma (BL) cells taken directly from patients express only a single latency protein, designated EBNA-1 (EBV associated nuclear antigen). However, lymphoblastoid cell lines (LCLs) derived from these same patients express all six latency EBNA proteins (EBNA-1,-2,-3,-4,-5,-6) and the latent membrane protein (LMP). Examination of biopsies from nasopharyngeal carcinomas (NPC) for expression of these seven latency genes demonstrated only EBNA-1. These observations suggest that there is a tumor-specific regulation of EBV genes in that BL and NPC tumor cells differ in gene expression from LCLs derived in vitro (34,53).

In contrast to EBV, herpes simplex virus (HSV) types 1 and 2 are associated primarily with infectious diseases. While HSV-2 has been suggested as a cofactor in cervical cancer, the primary importance of these herpesviruses to cancer patients is HSV reactivation during immunosuppressive therapy. Such reactivations tend to become disseminated infections and are a significant cause of morbidity and mortality. Thus, the mechanism(s) of the maintenance of HSV latency and reactivation are of importance to cancer research.

The expression of HSV genes is a tightly and coordinately regulated process with the control of expression primarily at the level of transcription. The earliest (alpha) viral genes expressed in the infected cell have regulatory functions. Investigators have identified a latency associated transcript (LAT) which is partially complementary to an early HSV-1 transcriptional activator, the ICP0 (infected cell polypeptide) gene. Additional investigations have demonstrated the LAT transcript in the nuclei of human, murine and rabbit nerve ganglia latently infected with HSV-1. A spliced species of the LAT transcript has also been detected and the extent of its presence appears to be determined by both the strain of virus and the animal in which latent infection occurs. The failure,

thus far, to detect a protein transcript of LAT, as well as the presence of LAT in the cell nuclei, suggests that LAT may act by an anti-sense mechanism rather than by an effector protein product. Finally, the demonstration that deletion mutants missing the LAT sequences could establish a latent infection suggested that the LAT function was not involved in this step, but rather in the maintenance or reactivation steps (80).

The host immune system appears to mediate the transition from a reactivated herpesvirus infection back to the latent state. Class I major histocompatibility complex (MHC)-restricted, virus-specific cytotoxic T-lymphocytes (CTL) have been demonstrated to play a principal role in the recovery from virus infection. It was initially assumed that the CTL would recognize primarily structural virus proteins, such as viral glycoproteins, present on the infected cell surface. However, analysis of the target antigens on HSV-1-infected cells has suggested that a significant fraction (approximately 20-35%) of CTL recognizes nonstructural HSV early (alpha) gene products. This is in accord with findings of others demonstrating CTL responses to internal antigens of influenza, Epstein-Barr virus and mouse cytomegalovirus. In all of these cases, the internal viral polypeptides are not known to be found on the surface of infected cells. However, since the CTL response recognizes processed antigens, it is hypothesized that the processed form of these antigens may be present on the cell surface. These observations suggest that it may be to the host's advantage to recognize infected cells at the time that they express viral early gene polypeptides and before the synthesis of viral structural protein (12).

A new human herpesvirus, human herpesvirus 6 (HHV-6), was discovered in 1986. Retrospective analysis of sera suggest that this virus has been present in the human population for many years. HHV-6 has been shown to be associated with exanthem subitem (roseola infantum) in young children. Its role in adult diseases or syndromes, if any, is under active investigation. On the basis of studies of antibody prevalence, it appears that most individuals are infected by three years of age. After that time, the percent of individuals with a positive antibody titer declines, suggesting that unlike HSV-1 and HSV-2 which have frequent recurrences, HHV-6 does not frequently recur. However, investigators have identified a syndrome in adults involving a mild, afebrile illness with nonspecific symptoms in which the characteristic clinical feature is the presence early in the illness of enlarged, bilateral, non-tender, anterior and posterior cervical nodes. These enlarged lymph nodes persist for several weeks to months. Individuals with these symptoms had falling IgM and high IgG antibody titers to HHV-6, suggesting that these adults had a recent primary infection with HHV-6. Additional studies compared individuals who were seropositive or seronegative for human immunodeficiency virus type 1 (HIV-1) infection for serological evidence of infection with CMV, EBV and HHV-6. The results suggested that individuals seropositive for HIV-1 infection had a higher prevalence and higher titers of antibodies to CMV and EBV than did HIV-1-seronegative individuals. In contrast, infection with HHV-6 did not differ between individuals who were seropositive or seronegative for HIV-1 (5).

Several animal herpesviruses causing neoplasias in their natural hosts may serve as models for herpesvirus oncogenesis in humans. Two recent findings with herpesvirus saimiri suggest that this virus may have derived two of its functions from host genes. One of these functions is an enzyme involved in DNA synthesis. DNA sequences for the enzyme dihydrofolate reductase (DHFR) have been found in herpesvirus saimiri. These DNA sequences share 83 percent positional identity in

amino acid sequence with the human DHFR gene. Many herpesviruses are known to encode enzymes for nucleotide and DNA synthesis, but this is the first time that homology with host sequences for such an enzyme has been found. By contrast, RNA tumor viruses have long been known to derive genetic material from their host cells (13).

The second host cell function found in herpesvirus saimiri is involved in the synthesis of proteins from a messenger RNA (m-RNA) template. Marmoset T-lymphocytes transformed by herpesvirus saimiri have been shown to contain the virus encoded U RNAs (the term U RNA is derived from eukaryotic RNA; the virus encoded U RNAs are designated HSURs). These U RNAs are the RNA components of small nuclear ribonucleoproteins (snRNPs), a class of structurally related RNA-protein complexes found in the nuclei of eukaryotic cells. snRNPs are involved in mRNA maturation. Analysis of deletion mutations suggests that neither the HSUR or DHFR genes are necessary for virus replication or immortalization. Because of the large genome of herpesviruses, it is possible for them to carry, as viral genes, functions that other viruses must derive from their host cells. The fact that the virus has acquired and retained these sequences suggests that they have a role in virus pathogenesis. While the host cell may have similar genes, the presence of viral genes under the control of viral promoters may allow the virus to inhibit the cellular counterpart gene while retaining the ability to have the function using the virus sequences. Future studies will focus on a role for these genes in maintenance of latency and in transformation (13).

Other studies of herpesvirus saimiri have demonstrated that virus strains can be classified into three groups based on DNA homology in the region of the virus genome associated with transformation. The members of each of these groups have different levels of oncogenicity in animals. When these strains were studied for their ability to induce interleukin 4 (IL-4) in transformed cells, a correlation was found between oncogenicity in rabbits and the ability to induce IL-4. Additional studies will investigate the biological basis for this observation (44).

Adenoviruses

Adenoviruses are a group of medium sized DNA viruses whose oncogenicity has been demonstrated by their ability to transform epithelial cells in vitro and to induce tumors in newborn rodents, in vivo. Extensive analysis of the adenovirus DNA sequences has demonstrated that two subregions, E1A and E1B, of the E1 genome region contain the genetic information necessary for transformation. The E1A and E1B regions also play important roles in the replicative cycle of the virus where they serve as regulators of viral and host-cell gene transcription. Investigators have constructed a variety of point, deletion, and missense mutations in the E1 region, and studied the effects of these alterations on the transformation and transcriptional regulatory functions of the E1 genes.

As part of such mutational analyses, investigators have compared the ability of regulatory genes from other viruses to substitute for mutated or deleted regulatory genes of adenoviruses. They have demonstrated that the immediate early genes of two herpesviruses, cytomegalovirus and pseudorabies virus, can substitute for a defective adenovirus E1A gene in transactivating the E1B gene. Because E1A can transactivate a broad spectrum of viral and cellular genes which do not appear to share common responsive DNA sequences, it has been hypothesized that E1A acts by up-regulating the activity of host-cell transcription factors

rather than by binding to specific DNA sequences. This hypothesis is supported by the observations that a variety of viruses in addition to adenoviruses, e.g., simian virus 40 (SV40) and pseudorabies, also possess broad transactivating capabilities. Additional studies have defined the TATA box in the promoter sequences of the E1B gene as the site for interaction with the E1A gene product. Further studies have demonstrated that this same TATA box is involved in transactivation of E1B by the pseudorabies virus immediate early gene. Demonstration that viruses as diverse as adenovirus and pseudorabies virus may use common mechanisms to affect viral and host-cell genes should facilitate investigations of virus-host interactions by allowing investigators to apply observations from one virus-host system to another (3,54).

Other functional studies of the E1A protein have demonstrated that it can bind to at least 11 host-cell polypeptides. In order to determine the function of these virus-host complexes, E1A deletion mutants were studied for retention of two functions: transformation and formation of protein-protein complexes. It was noted that two regions of the E1A protein, amino acids 1-76 and amino acids 120-127, were required for transformation. The cellular proteins which bound to these regions were identified as a 105 kilodalton (kD) protein (binding to E1A amino acid residues 30-60 and 120-127), a 107 kD protein (binding to residues 1-76) and a 300 kD protein (binding to residues 120-127). The 105 kD protein had several characteristics similar to that of the retinoblastoma (Rb) protein. The Rb protein is encoded by the Rb susceptibility gene. Loss or inactivation of both copies of this gene in humans may predispose an individual to retinoblastoma (an eye cancer in which the gene was first identified) and to some forms of bone and lung cancer. Studies using precipitation of protein complexes from cell lysates demonstrated the presence of intracellular complexes containing the E1A polypeptide and the Rb gene product. Similar complexes have subsequently been found between the SV40 T-antigen and the Rb polypeptide and between the E7 transforming polypeptide of papillomaviruses and the Rb 105 kD polypeptide. These findings represent the first demonstration of interactions between viral oncogenes and cellular anti-oncogenes and may form the basis for new hypotheses of viral transformation (42).

A very important aspect of virus gene regulation is the shift from synthesis of early to late virus proteins. The transformed state is characterized by the synthesis of only early gene products and a failure to make late gene products. Adenovirus late transcription is characterized by the very effective translation of late virus proteins, in part resulting from inhibition of transport of cellular messages from the nucleus to the cytoplasm. Additionally, all of the late virus proteins share a common 5′ non-coding sequence designated as the tripartite leader. These 5′ sequences seem to be both preferentially transcribed and to have enhanced translation. The tripartite leader appears to enhance translation by eliminating the requirement for a cellular transcription complex, the cap binding protein complex. Normally, the cap binding protein complex binds mRNA cap structures in the presence of ATP and stimulates protein synthesis. The tripartite leader alone was shown to permit translation of a heterologous hepatitis B virus surface antigen protein in the absence of an intact cap binding protein complex in uninfected cells and does not require the participation of any adenovirus gene. Several other viruses also encode mRNAs which have an intrinsic ability to initiate translation more efficiently than cellular messages. Most of these are RNA viruses; it is not yet clear whether all of these viruses use a similar type of mechanism to enhance the translation of viral messenger RNAs (64).

Although the majority of human neoplasms arise in epithelial cells, study of epithelial cell transformation has been hindered by the inability to readily grow primary epithelial cells in culture. A method has been developed which has the potential for providing investigators with epithelial cell lines. The expression of a single transcript from the E1A gene, the 243R protein (12S gene), immortalizes primary rodent epithelial cells and enables them to proliferate in the presence or absence of serum. The C-terminal region of the 243R polypeptide was shown to induce a growth factor which immortalizes the epithelial cells. A retrovirus vector expressing this gene has been developed. While these immortalized cell lines are non-tumorigenic, they can be completely transformed by the ras oncogene. This system has the potential for allowing the study of epithelial cell transformation by a variety of oncogenic viruses (42).

The E3 region of adenoviruses has recently been shown to play a key role in transformation. This early region encodes at least seven transcripts. Three of these transcripts are involved in altering cell properties such that the host's response to virus-transformed cells is affected. The gp19 transcript has been localized to the endoplasmic reticulum where it binds to class I MHC antigens and prevents their transport to the cell membrane. In this way, it protects transformed cells from lysis by cytotoxic T-cells. The 14.7 kD protein has been shown to protect cells from cytolysis by tumor necrosis factor. The synthesis of the 14.7 kD protein is induced by the E1A proteins. Finally, the 10.4 kD protein appears to down-regulate the synthesis of epidermal growth factor in the early stages of adenovirus infection and thus, may alter the number and/or type of cellular factors present in the cell and may be involved in virus-host interactions. Thus, while the E3 gene is not essential for virus replication in vitro, its functions in vivo appear to be highly relevant to the process of adenovirus transformation (7,18,85).

On April 25-26, 1988, this Branch component co-sponsored with the Virology Branch, National Institute of Allergy and Infections Diseases (NIAID), a workshop entitled "Mechanisms of Pathogenic Diversity of Epstein-Barr Virus." The participants identified a number of gaps in our knowledge of EBV pathogenesis and oncogenesis on which future research should focus. After programmatic evaluation, an RFA concept entitled "New Approaches to Studying Epstein-Barr Virus Oncogenesis" was developed and subsequently approved at the October 1988 meeting of the DCE Board of Scientific Counselors. The receipt date for applications to this RFA is August 3, 1989 with funding of meritorious applications to take place in FY90.

In summary, several new aspects of virus-host interactions in herpes- and adenovirus oncogenesis have been identified. However, further studies must be done in order to elucidate the mechanisms by which these viruses cause oncogenesis in vivo. The final goal of such studies is the control and reversal of this process.

DNA VIRUS STUDIES I

GRANTS ACTIVE DURING FY89

Investigator/Institution/Grant Number	Title
1. AURELIAN, Laure University of Maryland (Baltimore) 5 R01 CA 39691-03	Transformation by Restriction Fragments of HSV DNA
2. BABISS, Lee E. Rockefeller University 1 R29 CA48707-01	Transformation Progression and Adenovirus 5 Gene Regulation
3. BERK, Arnold J. University of California (Los Angeles) 5 R37 CA 25235-11	Biosynthesis of Adenovirus Early RNAs
4. BERK, Arnold J. University of California (Los Angeles) 2 R01 CA 41062-04	Transcription Stimulation by Adenovirus E1A Protein
5. BROWN, Nathaniel A. North Shore University Hospital (Manhasset, New York) 5 R01 CA 35536-07	Clonal Virulence Features of the EBV Terminal Region
6. CALNEK, Bruce W. Cornell University (Ithaca) 5 R01 CA 06709-27	Studies on the Avian Leukosis Complex
7. CARLIN, Cathleen R. St. Louis University 1 R01 CA 49540-01	EGF Receptor Down-Regulation by Adenovirus
8. CHANG, Robert S. University of California (Davis) 5 R01 CA 43051-03	Epstein-Barr Virus and Nasopharyngeal Carcinoma
9. CHINNADURAI, Govindaswamy St. Louis University 5 R01 CA 31719-08	Genetic Analysis of Adenovirus 2 Early Genes
10. CHINNADURAI, Govindaswamy St. Louis University 5 R01 CA 33616-10	Adenovirus LP Locus: Role in Oncogenic Transformation

11.	COOPER, Neil R. Scripps Clinic and Research Foundation 5 R01 CA 14692-17	Humoral Immunity to Viruses and Virus-Infected Cells
12.	COURTNEY, Richard J. Louisiana State University Medical Center (Shreveport) 5 R01 CA 42460-05	Studies of Purified Herpes Simplex Virus Glycoproteins
13.	DESROSIERS, Ronald C. Harvard University 5 R01 CA 31363-08	Molecular Basis for Herpesvirus Saimiri Oncogenicity
14.	EGGERDING, Faye A. St. Vincent Medical Center (Los Angeles) 7 R01 CA 25545-07	Regulation of Adenovirus 2 Transcription
15.	GALLOWAY, Denise A. Fred Hutchinson Cancer Research Center 5 R01 CA 26001-11	Herpesvirus Expression in Transformation and Latency
16.	GALLOWAY, Denise A. Fred Hutchinson Cancer Research Center 5 R01 CA 35568-06	Molecular Studies on Herpes- and Papillomavirus Proteins
17.	GAYNOR, Richard B. University of California (Los Angeles) 5 R01 CA 30981-08	Transcriptional Regulation by the Adenovirus E1A Protein
18.	GOODING, Linda R. Emory University 5 R01 CA 48219-02	Mechanism of Adenovirus-Induced TNF Resistance
19.	GREEN, Maurice St. Louis University 5 R01 CA 29561-32	Biochemistry of Animal Virus Multiplication
20.	HARDWICK, Jan Marie Johns Hopkins University 5 R01 CA 43532-03	Epstein-Barr Virus: Regulation of Gene Functions
21.	HAYWARD, Gary S. Johns Hopkins University 2 R37 CA 22130-12	Structure and Regulation of Human Herpesvirus Genomes
22.	HAYWARD, Gary S. Johns Hopkins University 5 R01 CA 28473-09	Cellular Transformation by DNA of Human Herpesvirus

23.	HAYWARD, S. Diane Johns Hopkins University 5 R37 CA 30356-08	EBV Genome Expression: Localization of Specific Functions
24.	HAYWARD, S. Diane Johns Hopkins University 2 R01 CA 42245-04	Regulation of Replication and Latency by EBV EBNA 1
25.	HOLMES, Edward W., Jr. Duke University 5 R01 CA 47631-02	Retroviral Anti-Sense RNA: Cellular and Viral Responses
26.	HORWITZ, Marshall S. Yeshiva University 2 R01 CA 11512-20	Adenovirus DNA Synthesis and Polypeptide Assembly
27.	HUANG, Eng-Shang University of North Carolina (Chapel Hill) 5 R01 CA 21773-10	Cytomegaloviruses and Human Malignancy
28.	HYMAN, Richard W. Pennsylvania State University Hershey Medical Center 5 R01 CA 16498-14	Malignancy and DNA Homology among the Herpesviruses
29.	ISOM, Harriet C. Pennsylvania State University Hershey Medical Center 5 R01 CA 23931-12	Regulation of Differentiation in Hepatocytes in Vitro
30.	IZANT, Jonathan G. Yale University 5 R01 CA 47629-02	Enhancement and Modulation of Anti-Sense RNA Activity
31.	JARIWALLA, Raxit J. Linus Pauling Institute 5 R01 CA 42467-03	Role of Transforming HSV-2 DNA Sequences
32.	JONES, Clinton J. University of Mississippi Medical Center 5 R29 CA 47872-02	Mechanistic Approaches to HSV-2 Induced Transformation
33.	KIEFF, Elliott D. Brigham & Women's Hospital (Boston) 5 R35 CA 47006-03	Molecular Biology of Epstein- Barr Virus Infection
34.	KLEIN, George Karolinska Institutet 5 R01 CA 28380-08	EBNA and Other Viral Products in EBV Transformed Cells

35. KLEIN, George
Karolinska Institutet
5 R01 CA 30264-08
Immune Effector Mechanisms in EBV-Carrying Patients

36. KNIPE, David M.
Harvard University
5 R37 CA 26345-10
Genetics of Herpesvirus Transformation

37. KNIPE, David M.
Harvard University
1 R13 CA 50302-01
Fourteenth International Herpesvirus Meeting

38. LEVINE, Arnold J.
Princeton University
1 R01 CA49271-01
The Proteins and Gene Functions of Epstein-Barr Virus

39. LEWIS, James B.
Oncogen
(Seattle)
5 R01 CA 29600-08
Functions of Adenovirus Proteins in Transformation

40. LEWIS, James B.
Oncogen
(Seattle)
7 R01 CA 39636-03
Adenoviral Oncogene Expression and Transformation

41. MARTIN, Terence E.
University of Chicago
5 R01 CA 48189-02
Effects of HSV on Nuclear Structure and mRNA Processing

42. MATHEWS, Michael B.
Cold Spring Harbor Laboratory
5 P01 CA 13106-18
Cold Spring Harbor Laboratory Cancer Research Center

43. MC DOUGALL, James K.
Fred Hutchinson Cancer Research Center
5 R01 CA 29350-08
The Biology of Transformation by Herpesvirus

44. MEDVECZKY, Peter G.
University of Massachusetts
(Worcester)
2 R01 CA 43264-04
Growth Factors and Herpesvirus Saimiri Induced Lymphomas

45. MILLER, I. George, Jr.
Yale University
2 R37 CA 12055-18
Studies of Epstein-Barr Virus

46. NEMEROW, Glen R.
Scripps Clinic and Research Foundation
5 R01 CA 36204-05
Infection of B Lymphocytes by Epstein-Barr Virus

47. PADMANABHAN, Radha K.
University of Kansas
Medical Center
5 R01 CA 33099-05

Structure and Functional Analysis of Adenovirus Genomes

48. PAGANO, Joseph S.
University of North Carolina
(Chapel Hill)
2 P01 CA 19014-12

Viral Oncogenesis and Latency

49. PEARSON, Gary R.
Georgetown University
5 R01 CA 39617-06

Epstein-Barr Virus-Specific Antigens

50. PEARSON, Gary R.
Georgetown University
1 R13 CA 48657-01

Third International Symposium on Epstein-Barr Virus and Associated Diseases

51. PEARSON, George D.
Oregon State University
5 R01 CA 17699-13

Replication of an Oncogenic Virus

52. PRUSOFF, William H.
Yale University
5 R01 CA 05262-29

Iododeoxyuridine, Iodo-DNA and Biological Activity

53. RAAB-TRAUB, Nancy J.
University of North Carolina
(Chapel Hill)
5 R01 CA 32979-05

EBV Expression in Nasopharyngeal Carcinoma

54. RAPP, Fred
Pennsylvania State University
Hershey Medical Center
5 P01 CA 27503-10

DNA Viruses and Neoplasia

55. RAPP, Fred
Pennsylvania State University
Hershey Medical Center
5 R01 CA 34479-07

Latency and Transformation by Herpesviruses

56. RASKA, Karel, Jr.
Robert Wood Johnson
Medical School
(Piscataway, New Jersey)
5 R01 CA 21196-12

Adenovirus T and Surface Antigens and Tumorigenicity

57. REKOSH, David M.
State University of New York
(Buffalo)
5 R01 CA 25674-09

Adenovirus Early Gene Function and DNA Replication

58.	RICCIARDI, Robert P. Wistar Institute of Anatomy and Biology 5 ROl CA 29797-08	Organization and Expression of Adenovirus Genes
59.	ROIZMAN, Bernard University of Chicago 5 R37 CA 08494-23	Mechanisms of Viral Infection in Relation to Cancer
60.	ROIZMAN, Bernard University of Chicago 5 POl CA 19264-13	UCCRC: Viral Oncology Program
61.	ROIZMAN, Bernard University of Chicago 5 R35 CA 47451-02	Molecular Biology of Herpes Simplex Viruses
62.	ROUHANDEH, H. Southern Illinois University 5 ROl CA 38678-02	Transforming Sequences of Yaba Virus DNA
63.	SCHAFFER, Priscilla A. Dana-Farber Cancer Institute 5 ROl CA 20260-13	Immediate-Early Genes of HSV
64.	SCHNEIDER, Robert J. New York University Medical Center 5 ROl CA 42357-03	Translational Regulation of Adenovirus Gene Expression
65.	SHENK, Thomas E. Princeton University 5 R37 CA 38965-06	Structure and Function of DNA Tumor Virus Genomes
66.	SILVERSTEIN, Saul J. Columbia University (New York) 5 ROl CA 17477-15	Molecular Biology of Herpesvirus
67.	SIXBEY, John W. St. Jude Children's Research Hospital 5 ROl CA 38877-05	Epstein-Barr Virus Expression in Normal Human Epithelium
68.	SPEAR, Patricia G. Northwestern University (Chicago) 5 R37 CA 21776-13	Herpesvirus Gene Expression in Transformed Cells
69.	SPECK, Samuel H. Dana-Farber Cancer Institute 2 ROl CA 43143-04	Viral Transcription in EBV Transformed Human B Cells

70. SPECTOR, Deborah H.
University of California
(San Diego)
5 R01 CA 34729-07

Human CMV, Cell-Related DNA, Oncogenes and Kaposi's Sarcoma

71. SPECTOR, Deborah H.
University of California
(San Diego)
1 R13 AI/CA 27197-01

Second International Cytomegalovirus Workshop

72. STRAIR, Roger K.
Yale University
7 R29 CA49047-02

Isolation of a Human "E1A-Like" Factor

73. STROMINGER, Jack L.
Dana-Farber Cancer Institute
5 P01 CA 21082-13

Molecular Basis of Viral Oncogenesis

74. SULLIVAN, John L.
University of Massachusetts
(Worcester)
5 R01 CA 39653-04

Lymphotropic Herpesvirus of Cottontail Rabbits

75. SUMMERS, Jesse
American Association for Cancer Research, Inc.
(Philadelphia)
1 R13 CA 50482-01

The Role of DNA Viruses in Human Tumors

76. TANAKA, Akiko
Tampa Bay Research Institute
1 R01 CA 50523-01

Marek's Disease Virus: Analysis of Latent Genes

77. THORLEY-LAWSON, David A.
Tufts University
2 R37 CA 31893-08

Epstein-Barr Virus Membrane Antigen

78. TIBBETTS, Clark J.
Vanderbilt University
5 R01 CA 34126-07

Adenovirus Genome Expression: Physical Mapping Studies

79. VELICER, Leland F.
Michigan State University
(East Lansing)
5 R01 CA 45479-03

Oncogenic Herpesvirus Secretory Glycoprotein Analysis

80. WAGNER, Edward K.
University of California
(Irvine)
2 R37 CA 11861-20

Control of Viral RNA Synthesis in Herpesvirus Infection

81.	WAGNER, Edward K. University of California (Irvine) 1 R13 CA 47733-01	Thirteenth International Herpesvirus Workshop
82.	WEISSMAN, Sherman Yale University 2 P01 CA 16038-16	Program on the Molecular Basis of Viral Transformation
83.	WILLIAMS, James F. Carnegie-Mellon University 5 R01 CA 21375-12	Genetic Analysis of Adenoviruses
84.	WILLIAMS, James F. Carnegie-Mellon University 5 R01 CA 32940-08	Type 12 Adenovirus Transformation-Defective Mutants
85.	WOLD, William S. St. Louis University 5 R01 CA 24710-11	Adenovirus 2 Coded Early Glycoprotein
86.	YATES, John L. Roswell Park Memorial Institute (Buffalo) 5 R01 CA 43122-03	The Functions of Epstein-Barr Virus Nuclear Antigen 1

CONTRACTS ACTIVE DURING FY89

Investigator/Institution/Contract Number		Title
87.	HAMPAR, Berge Biomolecular Technologies N43-CP-95634	Antibody Probes for Human Lymphotropic Virus (HBLV)
88.	HAMPAR, Berge Biomolecular Technologies N43-CP-95642	Molecular Probes for Human B-Lymphotropic Virus (HBLV)
89.	LEWIS, Marcia BIOS Corporation N43-CP-95631	Molecular Probes for Oncogenic Herpesviruses (EBV)
90.	NAGHASHFAR, Zohreh Molecular Diagnostic Systems, Inc. N43-CP-95633	Monoclonal Antibodies to Epstein-Barr Virus Peptides

SUMMARY REPORT

DNA VIRUS STUDIES II

The DNA Virus Studies II component of the Branch involves the investigation of the two major classes of mammalian small DNA tumor viruses: papillomaviruses and polyomaviruses. In the component, there are 111 research grants and contracts with an estimated total funding of 21 million dollars. These include traditional research grants (R01), program project grants (P01), conference grants (R13), outstanding investigator grants (R35), the method to extend research in time (MERIT) awards (R37), new investigator grants (R23), and first independent research support and transition (FIRST) awards (R29). In addition, there are three Phase II small business innovative research (SBIR) contracts. The major research emphasis of this area is the elucidation of the molecular events leading to the initiation and maintenance of cellular transformation by the small DNA tumor viruses and the determination of the role of these viruses in the etiology of human cancers. In terms of scientific areas, 33% deal with the biochemical properties and mechanisms of action of viral proteins; 32% deal with the structure and expression of viral genes; 20% deal with the expression and function of cellular genes, including known proto-oncogenes that are involved in the transformation process; 10% deal with the potential etiological relationship of small DNA viruses to human cancers; 3% deal with the host immune response to these tumor viruses; and 3% deal with basic biochemical and physiological studies which use the small DNA tumor viruses as model systems. In terms of the viruses being studied, 33% of the grants involve the simian virus 40 (SV40); 25% concern human or animal papillomaviruses; 15% deal with viral or cellular oncogenes or related systems; 15% relate to mouse polyomavirus; 6% relate to human polyomaviruses; and 6% deal with other DNA or RNA viruses. Representative studies involving these classes of viruses are described below.

Anti-Oncogenes and Viral Transforming Proteins

It has been a landmark year in the study of the small DNA viruses. Important discoveries have been made involving the interaction of viral transforming proteins with cellular proteins which are the products of known and suspected anti-oncogenes. The first recognized anti-oncogene, Rb, was originally identified as a gene whose loss as a functional entity led to the development of retinoblastoma (an eye cancer) in children. It was thus named a cancer suppressor gene or an anti-oncogene to distinguish it from other previously identified cellular oncogenes whose presence at aberrantly high levels or in an activated mutant form can transform cells in culture. The loss of functional Rb genes have also been implicated in the etiology of some lung and bone cancers.

The major advance this year has been the discovery that all the known small and medium sized DNA tumor viruses have viral transforming proteins which bind to and presumably interact with the product of the Rb anti-oncogene. The Rb protein binds to the large T-antigens of simian virus 40 (SV40), mouse polyomavirus, hamster polyomavirus, human JC virus and human BK virus. In addition, binding has been detected to the E7 oncoprotein of human papillomavirus type 16 as well as to the adenovirus E1A oncoprotein. The association between viral transforming proteins and a cellular anti-oncogene suggests that this interaction is involved in transformation by these DNA tumor viruses. The Rb protein is known to be a DNA binding phosphoprotein which appears to down-regulate host cell DNA synthesis

in cells. The simplest hypothesis for the mechanism of transformation by these viruses is one where the association of the Rb protein with a viral transforming protein inactivates Rb, thereby allowing continued DNA replication in the cell. All the viral transforming proteins which bind Rb are also nuclear proteins which is consistent with this hypothesis. One of these viral proteins, the polyoma-virus large T-antigen, can only immortalize cells in culture. This result suggests that the influence of the Rb interaction may be confined to the immortalization step of the transformation process. Detailed studies on the interaction between Rb and one tumor virus transforming protein, the SV40 large T-antigen, is discussed in the following SV40 section (56,58,59, DNA I component reference 42).

A second advance in the anti-oncogene-viral protein area is the preliminary identification of a new anti-oncogene, the gene for the p53 protein. This cellular protein was discovered ten years ago through its binding to SV40 T-antigen. Interest in studying the p53 protein and its gene has been substantial because it also binds to the adenovirus E1B protein and it is found in some animal tumors at elevated levels. These non-viral tumors arise in animals exposed to certain chemical carcinogens or in transgenic mice which carry the SV40 T-antigen transgene. The p53 protein is a nuclear phosphoprotein whose function in the cell is not understood. Recent data suggests that it may also have a regulatory role in the cell cycle. The binding of p53 to large T-antigen markedly reduces the ability of T-antigen to support viral DNA replication in vitro. This binding also causes a reduction in the ability of T-antigen to bind to and unwind viral DNA. Genetic studies also suggest that p53 binding may be required for the tumorigenicity of SV40 T-antigen in animals (74,75,98).

The p53 protein was initially felt to be a cellular proto-oncogene. In early transfection experiments, p53 DNA was able to cooperate with the ras oncogene to transform primary cells in culture. Recent studies, however, found that the p53 DNAs used in these transfection experiments were mutant p53's. It is now known that wild-type p53 DNA does not immortalize primary cells nor is it able to cooperate with ras to transform these cells. This wild-type p53 DNA also has the ability to suppress transformation by other oncogenes. For example, wild-type p53 DNA can inhibit transformation by the potent adenovirus E1A and ras combination. The few transformed cells that arose in this three-way transfection did not contain active p53 genes. Mutations at many locations in the p53 DNA can apparently activate the p53 gene for transformation. This fact is consistent with the notion that this activation actually represents a loss of function as would be expected from an anti-oncogene. It is hypothesized that SV40 large T-antigen binding to p53 may also represent an interaction that inactivates this protein thereby contributing to the transformation process (55,84).

Simian Virus 40 (SV40)

SV40 is a major focus of transformation research in this Branch component. This small DNA tumor virus was isolated from monkeys and quickly became a popular model system to study viral transformation of cells in culture and tumorigenesis in susceptible rodents. Previous research has demonstrated that the transformation properties of SV40 are associated with the viral gene that encodes the large T-antigen. This protein dominates both the lytic and transforming interactions of SV40 with the host cell. It provides many biochemical functions for viral infection, including modulation of DNA transcription and replication,

and the production of cell surface antigens. In contrast to a growing number of viral oncogenes that require multiple proteins acting cooperatively to produce a fully transformed phenotype, the large T-antigen alone can convert primary cells into tumorigenic cell lines. All of the viral functions required to immortalize cells and to confer the transformed phenotype reside in this protein. Thus, the gene for the large T-antigen and its protein product have become primary areas of investigation in SV40-mediated transformation studies.

Among the small DNA tumor viruses, the interaction between the Rb protein and SV40 large T-antigen is the best studied of such interactions. Investigators supported by this component originally identified the interaction and have further characterized it. The binding site of the Rb protein on the large T-antigen has been identified as amino acid residues 105 to 114. Similar binding sequences have been found in other viral transforming proteins. In fact, all the transforming proteins which bind Rb have extensive regions of amino acid sequence homology. A genetic analysis of the SV40 large T-antigen/Rb binding demonstrated that all non-Rb binding mutants of T-antigen were also transformation defective as measured by the focus formation and the soft agar growth tests. This result supports the hypothesis that the T-antigen/Rb interaction is necessary for cellular transformation. The Rb protein appears to be involved in regulating the initiation of the S phase (DNA synthesis) of the cell cycle. The phosphorylation state of the Rb protein appears to change during the cell cycle. The SV40 large T-antigen binds preferentially to underphosphorylated forms of the Rb protein. The relationship between Rb and the regulation of DNA synthesis as well as the mechanism by which SV40 large T-antigen modifies this regulation are areas of intense current investigation (56,58,59).

Another advance this year was the discovery of a biochemical function for the small t-antigen of SV40. This small protein shares some amino acid sequences with the large T-antigen, primarily in the amino terminal end. However, its role in both lytic infection and transformation has remained a mystery. Some, but not all, cell lines seem to require small t-antigen for full transformation to occur. Investigators have shown that the small t-antigen is a transactivating protein. It promotes the transcription of messenger RNA from RNA polymerase II and III promoter-enhancers. Large T-antigen also can apparently transactivate these promoters; however, it can also activate the viral late promoter which is unaffected by small t-antigen thus suggesting at least two independent transcription promoting domains in large T-antigen. Since small t-antigen cannot bind directly to DNA, this transactivating potential must be mediated through cellular transcription factors. A search for these factors is currently underway. It is tempting to conclude that this transactivating function must be involved in the transformation helping function of small t-antigen; however, genetic data from other investigators suggest that the transactivating and transforming activities of small t-antigen can be genetically separated. Mutant forms of the small t-antigen were found that are weak transformers (anchorage independence assay) but are fully competent to transactivate. More work is needed to fully understand the role of small t-antigen in transformation (57,79).

Viruses need a susceptible host in order to manifest their tumorigenic potential. SV40 requires an immunologically immature or immunosuppressed host for the development of tumors. Adult, immunocompetent animals do not usually develop tumors upon challenge with SV40 virus or with malignant cells which it has transformed. The molecular basis of this resistance to SV40-transformed cells has recently been shown to be a cell-mediated immune process. It provides an

excellent model of how a nuclear viral protein, such as the SV40 large T-antigen, can be the target of the immune system. Four distinct clones of cytotoxic T-lymphocytes (CTLs) have been isolated which can recognize and lyse cells transformed by SV40. The CTLs recognize four different amino acid sequences of the SV40 large T-antigen. These sequences have been precisely mapped using overlapping synthetic peptides corresponding to T-antigen sequences. Recognition by the CTLs requires processing (fragmentation) of the large T-antigen and presentation of the T-antigen fragments containing the immunogenic sequences on the surface of transformed cells by the H-$2D^b$ antigen of the MHC (mouse histocompatibility complex). Studies with mutant forms of the MHC antigen show that single amino acid changes in the H-$2D^b$ protein can abolish recognition of some of the CTL specific T-antigen epitopes by the CTLs. Minor changes in the MHC presenting antigen thus can inhibit its ability to present known epitopes on the cell surface. Such allelic differences in MHC antigens may represent a mechanism for a host's genetic resistance or susceptibility to viral oncogenesis. Studies such as these will be important in the development of appropriate preventive and control methods for virus-associated neoplasia (86,98,99).

Polyomaviruses

Another virus whose study is supported by this Branch component is mouse polyomavirus, which is similar to SV40. The SV40 and mouse polyomavirus virions are morphologically identical and contain nearly the same amount of DNA. However, the viral genomes are organized differently, particularly with respect to the early tumor antigen region which is responsible for transformation. Polyomavirus DNA codes for six proteins, including three tumor antigens: large T-antigen, middle T-antigen, and small t-antigen. The relationship among the tumor antigens with respect to cellular transformation is complex. The large T-antigen (which is localized in the nucleus) appears to be able to immortalize primary cells in culture, whereas the middle T-antigen (which becomes membrane bound) is able to induce the transformed phenotype in previously immortalized cell lines. In the past year, studies have focused on characterizing the structure and mechanism of action of these T-antigens. In particular, the intriguing observation that middle T-antigen can form tight complexes with the cellular protein products of the c-src family of proto-oncogenes has been vigorously pursued.

The c-src oncoprotein (pp60c-src) was the first cellular proto-oncogene product that was found to bind to a viral transforming protein, the polyomavirus middle T-antigen. It appears to mediate, in part, the transforming ability of middle T-antigen. The c-src protein is cytoplasmically active in contrast to the nuclear anti-oncogene proteins discussed above. The association of the c-src protein with middle T-antigen increases the tyrosine kinase activity of c-src protein by 20-fold. The mechanism that results in this increased activity has recently been elucidated. The kinase activity of the c-src protein appears to require phosphorylation of the tyrosine residue at position 416 and concomitant dephosphorylation of tyrosine 527. Subsequent biochemical analysis of c-src mutants demonstrated that tyrosine 527 is the major carboxyl terminal residue responsible for regulating the kinase activity of c-src in vivo and that residues aspartate 518 to proline 525 in c-src are the apparent binding site for middle T-antigen. The proximity of these binding sequences to tyrosine 527 suggests a model where T-antigen binding to this region prevents phosphorylation of tyrosine 527 by stearic hindrance, thus retaining the kinase activity of the c-src protein (86).

The phosphorylation of the middle T-antigen by the c-src protein seems to be a critical event during the induction of tumors in mice by polyomavirus. This conclusion was reached through studies on a mutant middle T-antigen where the normally phosphorylated tyrosine 315 residue is replaced with a phenylalanine which cannot be phosphorylated. This mutation induces a drastically reduced and altered tumor profile compared to the parental polyomavirus strain. The molecular basis of this response appears to be the requirement for phosphorylation of the middle T-antigen on tyrosine-315 for binding of the cellular "type I" phosphatidylinositol (PI) kinase to the middle T-antigen/c-src complex. This kinase produces a novel lipid, phosphatidylinositol-3-phosphate, when bound to this complex, thus implicating this lipid in oncogenesis by polyomavirus. This observation supports the findings of other investigators who have shown a correlation between PI kinase binding to middle T-antigen and the transformation competency of middle T-antigen (7,80).

Different strains of polyomavirus are known to have markedly different tumor profiles in susceptible mice. These viral strains, however, have identical growth and transformation properties in cell culture. The molecular cause of this difference was found to be changes in the protein coding sequences of the viral DNA rather than the regulatory sequences. Subsequent work has shown that the high and low tumor producing strains of virus differ only in three amino acids occurring in three viral proteins, the large and middle T-antigens and the major capsid protein. Recent work has eliminated the change in the middle T-antigen sequence as the primary mutation causing the tumorigenic differences between the two viruses. Thus, the critical change is either in the large T-antigen or the major capsid protein. Work is progressing to determine the role of each protein in tumorigenesis (7).

Papillomaviruses

Human papillomaviruses (HPV) are associated with cervical cancer and other malignancies of the anogenital tract. Previously, investigators had identified and classified HPV DNA from many premalignant, malignant, and metastatic lesions in humans. HPV types 16, 18, 31, 33, 35, 39 and 45 were usually associated with severe cervical dysplasias and carcinoma in situ, whereas HPV types 6, 11, 42, 43 and 44 were associated with milder lesions such as genital warts and mild dysplasias. In addition, established human cervical cancer cell lines, such as HeLa and CaSki, were found to possess integrated HPV-18 and -16 DNA, respectively. These data strongly suggest a role for HPVs in human cancer.

A new HPV model has recently been developed which may describe both viral latency/activation and the development of precancerous dysplasias. This model is based on transcription and in situ hybridization studies of HPV-associated cervical lesions. Briefly, the model proposes that in the basal and parabasal cells of the infected epithelium, HPV DNA transcription is kept at a low level sufficient for viral latency by the combined interaction of a viral protein, a product of the viral E2 gene, and various cellular factors. As the levels of the cellular factors change during differentiation, the block to HPV transcription is released allowing the increased synthesis of viral proteins and the eventual production of virions. Transformation of these differentiated cells does not occur because these cells have already lost the ability to divide.

Transformation to a dysplastic state occurs only in tissue levels that are still competent for cell division, the basal and parabasal cells, when a rare event occurs that disrupts the block to viral transcriptions allowing the high level transcription of the viral transforming genes E6 and E7. The most common lesion that can remove this block appears to be integration of the viral DNA into the host genome in a manner that physically disrupts the E2 gene, thus preventing the production of an inhibitory protein. To test this model, many experiments, involving areas such as the identification of the cellular differentiation factors, will be needed (17,48,92).

One host factor which appears to effect human papillomavirus transcription in vivo has been identified. These studies used the nude mouse xenograft model in which human tissue fragments infected with papillomavirus are placed under the renal capsule of immunodeficient mice. These infected fragments can subsequently develop into tissue masses having all the histological properties of human cervical condylomas and mild dysplasias. Recent studies have demonstrated that administration of the sex hormone, beta-estradiol, to the mouse host has a profound effect on the growth of the infected human tissues (induced condylomas) in situ. Beta-estradiol-treated tumor masses in the mice were often 3 to 4 times larger than untreated tumors, grew two- to threefold faster, had higher levels of viral RNA and DNA and contained many isolatable virions. Other sex hormones such as progesterone, methyltestosterone and oral contraceptives had comparable effects on experimental condylomas. The mechanism of action of these sex hormones on HPV-infected tissues probably involves direct stimulation of these tissues through the hormone receptor naturally present in these human cells (a nuclear protein which is known to stimulate cellular RNA synthesis). These hormones also suppress natural killer cell activity thereby helping assure that a minimal immune response is instituted against the transformed cells (49).

An RFA entitled "Animal Models to Human Papillomavirus-Associated Neoplastic Diseases" was issued in May 1988 with a receipt date of September 15, 1988. It was developed by program staff in response to recommendations of a workshop on "Prospects for Human Papillomavirus Vaccines and Immunotherapies." The goal of the RFA is the promotion of studies on the host immune mechanisms involved in the regression of HPV-associated lesions and on the molecular mechanisms which may lead to the progression of these lesions to carcinoma. Established animal models or new animal models were felt to be the best experimental systems to generate the needed data. Eleven applications were received in response to the RFA.

A workshop entitled "T-Antigens and Proto-Oncogene Interactions" was sponsored by this Branch component on April 17-18, 1989. The purpose of the workshop was to review progress in this rapidly developing field and to obtain recommendations on how the NCI can help promote this important research. A number of recommendations were received and are currently being evaluated for appropriate initiatives.

In summary, major advances have occurred this year in our understanding of the transformation mechanism of the small DNA tumor viruses. The discovery of the association of the Rb protein (an anti-oncogene product) with many of the transforming proteins of each of these viruses has helped to unify the study of these viruses and has suggested many interesting areas of research. It has linked transformation and tumorigenesis by these viruses with the mechanism of cancer development in several human tumors, since they may all share in common the inactivation of the Rb protein. In addition, work on these viruses has strongly

suggested that the gene encoding the cellular p53 protein may be a candidate anti-oncogene. The p53 gene appears to play a role in both viral and non-viral mechanisms of cancer development. In addition the mechanism of the cellular immune response against SV40-transformed cells has been elucidated at a molecular level. This work provides a basis for understanding how the immune system can use as targets viral proteins which function only in the cell nucleus.

DNA VIRUS STUDIES II

GRANTS ACTIVE DURING FY89

Investigator/Institution/Grant Number	Title
1. ALONI, Yosef Weizmann Institute of Science 5 R01 CA 14995-15	Control of Gene Expression in Tumor Viruses and Cells
2. ALWINE, James C. University of Pennsylvania 5 R01 CA 28379-09	Regulation of DNA Tumor Virus Gene Expression
3. ANDROPHY, Elliot J. New England Medical Center Hospital 5 R01 CA 44174-03	Characterization of Papillomavirus E6 Proteins
4. BASILICO, Claudio New York University 5 P01 CA 16239-15	Biosynthesis in Normal and Virus Transformed Cells
5. BASILICO, Claudio New York University 5 R35 CA 42568-04	Viral and Cellular Gene Expression and Growth Regulation
6. BECKMANN, Anna M. Fred Hutchinson Cancer Research Center 5 R01 CA 47619-02	Pathobiology of Anogenital HPV Infection
7. BENJAMIN, Thomas L. Harvard Medical School 5 R35 CA 44343-03	Natural and Unnatural Roles of the Polyoma HR-T Gene
8. BOTCHAN, Michael R. University of California (Berkeley) 5 R37 CA 30490-09	Regulatory Interactions Between Tumor Viruses and Cells
9. BOTCHAN, Michael R. University of California (Berkeley) 5 R01 CA 42414-04	Bovine Papillomavirus - Model Systems
10. BRADLEY, Margaret K. Dana-Farber Cancer Institute 5 R01 CA 38069-06	Nucleotide Binding Properties of SV40 Large T Protein
11. BRAUN, Lundy A. Brown University 5 R29 CA 46617-02	Oncogenes and Growth Factors in Human Gynecologic Cancers

12. BUTEL, Janet S.
Baylor College of Medicine
2 R01 CA 22555-12

Biological Properties of SV40 Early Proteins

13. BUTEL, Janet S.
Baylor College of Medicine
5 R01 CA 25215-11

Tumor Viruses, Oncogenes and Mammary Epithelial Cells

14. CARMICHAEL, Gordon G.
University of Connecticut Health Center
5 R01 CA 45382-03

Processing and Function of Polyoma RNA

15. CARROLL, Robert B.
New York University
5 R01 CA 20802-13

Biochemical and Functional Properties of the SV40 T-antigens

16. CHERINGTON, Van
Tufts University School of Medicine
1 R29 CA 44761-01

An Oncogene Sensitive Regulatory Event in Cellular Differentiation

17. CHOW, Louise T.
University of Rochester
5 R01 CA 36200-06

Human Papillomavirus Gene Expression

18. COLE, Charles N.
Dartmouth College
5 R01 CA 39259-05

The Molecular Biology of SV40 Large T-antigen

19. CONRAD, Susan E.
Michigan State University
5 R01 CA 37144-06

SV40-Induced Changes of Growth Regulation in Host Cells

20. CONSIGLI, Richard A.
Kansas State University
2 R01 CA 07139-26

Studies in Polyoma Transformed Cells: Virion Proteins

21. CRUM, Christopher P.
University of Virginia (Charlottesville)
5 R01 CA 47676-02

Pathology of Cervical Intraepithelial Neoplasia

22. DAS, Gokul C.
University of Texas Health Center at Tyler
5 R29 CA 47611-03

Regulation of Transcription in Polyoma Virus

23. DE BRITTON, Rosa M. C.
Gorgas Memorial Institute of Tropical Medicine, Inc.
5 R01 CA 42042-04

Human Papillomavirus and Cervical Cancer in Panama

24. DIMAIO, Daniel C. Yale University 5 R01 CA 37157-06	Analysis of Cell Transformation by Bovine Papillomavirus
25. DYNAN, William S. University of Colorado (Boulder) 5 R01 CA 44958-03	Functional Organization of the BK Virus Promoter/Enhancer
26. ECKHART, Walter Salk Institute for Biological Studies 5 R37 CA 13884-17	Viral Gene Functions and Regulation of Cell Growth
27. FARAS, Anthony J. University of Minnesota (St. Paul) 2 R01 CA 25462-10	Human Papillomaviruses and Malignant Disease
28. FLUCK, Michele M. Michigan State University 5 R01 CA 29270-08	Studies of the Integration of the Polyoma Virus Genome
29. FOLK, William R. University of Texas (Austin) 5 R01 CA 38538-05	Mammalian Cell Transformation by Oncogenic Viruses
30. FOLK, William R. University of Missouri 7 R01 CA 45033-02	Mechanism of Transformation by BK Virus
31. FOX, C. Fred University of California 1 R13 CA 49956-01	Conference on Papillomaviruses
32. FRISQUE, Richard J. Pennsylvania State University (University Park) 5 R01 CA 38789-05	A Molecular Approach to the Unique Biology of JC Virus
33. FRISQUE, Richard J. Pennsylvania State University (University Park) 5 R01 CA 44970-03	Human Polyomaviruses: Oncogenic Potential and Mechanisms
34. GARCEA, Robert L. Dana-Farber Cancer Institute 5 R01 CA 37667-06	Mechanisms in Polyomavirus Assembly
35. GREEN, Maurice St. Louis University 5 R01 CA 28689-08	Biochemical Functions of Papillomavirus Oncogenes
36. GURNEY, Elizabeth T. University of Utah 5 R01 CA 21797-08	Growth Control and Viral Gene Expression

37. HANAHAN, Douglas
University of California
5 R01 CA 47632-03

Oncogenesis by Papillomavirus DNAs in Transgenic Mice

38. HARRISON, Stephen C.
Harvard University
2 R01 CA 132002-18

Structure and Assembly of Viruses

39. HEARING, Patrick
State University of New York (Stony Brook)
5 R01 CA 44673-03

Analysis of a Polyomavirus Enhancer and Binding Protein

40. HERR, Winship
Cold Spring Harbor Laboratory
1 R13 CA 50494-01

Regulation of mRNA Transcription

41. HOWETT, Mary K.
Pennsylvania State University (Hershey Medical Center)
5 R01 CA 25305-10

Modulation of the Tumorigenicity of Transformed Cells

42. IMPERIALE, Michael J.
University of Michigan (Ann Arbor)
5 R01 CA 19816-14

Role of SV40 Gene A in Cellular Transformation

43. JENSON, A. Bennett
Georgetown University
1 R01 CA 50182-01

Antigenic Determinants of the Papillomavirus L1 Capsid Protein

44. KADISH, Anna S.
Albert Einstein College of Medicine of Yeshiva University
5 R01 CA 47630-02

Host Immunity to Genital Human Papillomavirus Infection

45. KELLY, Thomas J., Jr.
Johns Hopkins University
5 P01 CA 16519-15

Program on Molecular Biology of Viral Tumorigenesis

46. KELLY, Thomas J., Jr.
Johns Hopkins University
5 R01 CA 40414-05

Replication of the SV40 Genome

47. KHALILI, Kamel
Jefferson Medical College
Jefferson University
5 R29 CA 47996-02

Tissue Specific Transcription of JCV in Glial Cells

48. KREIDER, John W.
Pennsylvania State University (Hershey Medical Center)
5 R01 CA 42011-04

Human Papillomaviruses in Cervical Cancer

49. KREIDER, John W. Pennsylvania State University (Hershey Medical Center) 5 ROl CA 47622-02	Studies on Papillomavirus Host Interaction
50. LAIMINS, Laimonis University of Chicago 1 ROl CA 49670-01	HPV-18 Effects on Epithelial Cell Differentiation
51. LANCASTER, Wayne D. Wayne State University School of Medicine 7 ROl CA 32603-07	Role of Papillomavirus DNA in Cell Transformation
52. LANCASTER, Wayne D. Wayne State University School of Medicine 5 ROl CA 32638-09	Role of Papillomavirus in Cervical Neoplasia
53. LANFORD, Robert E. Southwest Foundation for Biomedical Research 5 ROl CA 39390-06	SV40 T-antigen: Model for Nuclear Transport of Protein
54. LEHMAN, John M. Albany Medical College of Union University 5 ROl CA 41608-04	Pathology of Neoplastic Transformation
55. LEVINE, Arnold J. Princeton University 5 ROl CA 38757-05	Viral Induced Tumorigenesis
56. LIVINGSTON, David M. Dana-Farber Cancer Institute 5 ROl CA 15751-16	Structure and Function of SV40 Non-Virion Proteins
57. LIVINGSTON, David M. Dana-Farber Cancer Institute 5 ROl CA 24715-11	Isolation and Function of Small SV40 T-antigen
58. LIVINGSTON, David M. Dana-Farber Cancer Institute 5 ROl CA 42339-03	Mechanism of Transformation by SV40 Large T-antigen
59. LIVINGSTON, David M. Dana-Farber Cancer Institute 1 ROl CA 49530-01	Repressor Control of SV40 Transformation
60. LIVINGSTON, David M. Dana-Farber Cancer Institute 1 POl CA 50661-01	Papovavirus Transforming Mechanisms

61. MANLEY, James L.
Columbia University
5 R01 CA 46121-03
Mechanism of Alternative Splicing of SV40 Pre mRNA

62. MANN, Kristine E.
University of Alaska (Anchorage)
1 R15 CA 41660-01
Enzymatic Activity of SV40 Tumor Antigen

63. MERTZ, Janet E.
University of Wisconsin (Madison)
5 R01 CA 37208-05
Involvement of T-antigen in SV40 Late Gene Expression

64. MOUNTS, Phoebe
Johns Hopkins University
5 R01 CA 35535-06
Analysis of Papillomavirus in Laryngeal Papillomatosis

65. MOUNTS, Phoebe
Johns Hopkins University
5 R01 CA 42089-03
Role of Human Papillomaviruses in Cervical Cancer

66. NORKIN, Leonard C.
University of Massachusetts
1 R01 CA 50532-01
Interaction of SV40 with MHC Class 1 Proteins

67. OZER, Harvey L.
Robert Wood Medical School
7 R01 CA 23002-13
Host Functions Related to Tumor Virus Infection

68. PALLAS, David C.
Dana-Farber Cancer Institute
5 R29 CA 45285-03
The Role of Cellular Proteins in Polyoma Transformation

69. PIPAS, James M.
University of Pittsburgh
5 R37 CA 40586-05
Genetic Analysis of the SV40 Large Tumor Antigen

70. PIRISI, Lucia A.
University of South Carolina
1 R29 CA 48990-01
Papillomavirus Transformation of Human Keratinocytes

71. PIWNICA-WORMS, Helen M.
Tufts University
1 R29 CA 50767-01
Role of Tyrosine Kinases in Middle T Antigen Transformers

72. POGO, Beatriz G.
Mount Sinai School of Medicine
5 R01 CA 29262-06
The Expression of Oncogenicity of Shope Fibroma Virus

73. POLLACK, Robert E.
Columbia University
5 R01 CA 38883-03
Tumor DNA Transformation of Diploid Cells: New Oncogenes

74. PRIVES, Carol L.
Columbia University
5 ROl CA 26905-10

Function/Expression of SV40 and Polyoma Tumor Antigens

75. PRIVES, Carol L.
Columbia University
5 POl CA 33620-06

Directed SV40 Mutation: Cell Molecular Consequences

76. RICCIARDI, Robert P.
Wistar Institute of Anatomy and Biology
5 ROl CA 44960-03

Role of BKV Enhancers in Virus Regulation and Cancer

77. ROBERTS, James M.
Fred Hutchinson Cancer Research Center
1 R29 CA 48718-01

Control of Viral Replication

78. ROBERTS, Thomas M.
Dana-Farber Cancer Institute
5 ROl CA 30002-08

Molecular Mechanisms of Polyoma-Induced Transformation

79. RUNDELL, Mary K.
Northwestern University
5 ROl CA 21327-12

Functions of the Simian Virus 40 Small T-antigen

80. SCHAFFHAUSEN, Brian S.
Tufts University
5 ROl CA 34722-07

Products of the Transforming Genes of Polyomavirus

81. SCHOOLNIK, Gary K.
Stanford University
5 ROl CA 43871-02

Cervical Neoplasia: Detection of HPV 16 Gene Products

82. SHAH, Keerti V.
Johns Hopkins University
5 ROl CA 42074-02

Outcome of Papillomavirus Infections of the Cervix

83. SHAH, Keerti V.
Johns Hopkins University
5 ROl CA 44962-02

Role of Polyomaviruses in Human Malignancies

84. SHENK, Thomas E.
Princeton University
5 POl CA 41086-04

Viral and Cellular Oncogenes: Mechanism of Action

85. SIMMONS, Daniel T.
University of Delaware
5 ROl CA 36118-05

Structure and Function of the SV40 Tumor Antigen

86. SMITH, Alan E.
Integrated Genetics, Inc.
5 ROl CA 43186-04

Mutagenesis of Papovavirus Transforming Proteins

87. SMITH, Janet L.
Gordon Research Conferences
1 R13 GM 39801-01
Diffraction Methods in Molecular Biology

88. SMOTKIN, David
University of Utah
5 R29 CA 47127-02
Human Papillomavirus Gene Expression in Cervical Cancer

89. SNAPKA, Robert M.
Ohio State University
5 R29 CA 45208-03
Aberrant Papovavirus Replication after Genotoxic Damage

90. SOMPAYRAC, Lauren M.
University of Colorado (Boulder)
5 R01 CA 34072-06
SV40 Deletion Mutants: Oncogenic Proteins

91. STEINBERG, Mark L.
City College of New York
5 R01 CA 27869-10
Oncogene Expression in SV40-Infected Keratinocytes

92. STOLER, Mark H.
The Cleveland Clinic Foundation
7 R01 CA 43629-04
Human Papillomavirus Expression in Squamous Neoplasia

93. SUBRAMANI, Suresh
University of California (San Diego)
5 R01 CA 44997-03
Mechanisms of Gene Regulation and Transformation in BK

94. SYRJANEN, Kari J.
University of Kuopio
5 R01 CA 42010-03
Natural History of Cervical HPV Infections

95. TACK, Lois C.
Salk Institute for Biological Studies
5 R01 CA 37081-06
SV40 T-antigen, Chromatin Structure and Viral Function

96. TEGTMEYER, Peter J.
State University of New York
(Stony Brook)
2 R37 CA 18808-15
Tumor Virus SV40: Protein Function and DNA Replication

97. TEGTMEYER, Peter J.
State University of New York
(Stony Brook)
5 P01 CA 28146-10
Tumor Virus-Host Interactions

98. TEVETHIA, Mary J.
Pennsylvania State University
(Hershey Medical Center)
5 R01 CA 24694-12
Mutagenesis of Specific Regions of the SV40 Genome

99.	TEVETHIA, Satvir S. Pennsylvania State University (Hershey Medical Center) 5 R37 CA 25000-12	Biology of SV40 Specific Transplantation Antigen
100.	TJIAN, Robert T. University of California (Berkeley) 5 R37 CA 25417-11	The SV40 Tumor Antigen
101.	TUREK, Lubomir P. University of Iowa 1 R01 CA 49912-01	Human Papillomavirus-16 Regulation in Cervical Cancer
102.	WALTER, Gernot F. University of California (San Diego) 5 R01 CA 36111-05	SV40 and Polyomavirus Transforming Proteins
103.	WATTS, Susan L. University of North Carolina (Chapel Hill) 5 R01 CA 42085-03	Cervical Neoplasia: HPV Epidemiology and Molecular Biology
104.	WETTSTEIN, Felix O. University of California (Los Angeles) 5 R37 CA 18151-14	Analysis of the Shope-Papilloma Carcinoma System
105.	WETTSTEIN, Felix O. University of California (Los Angeles) 5 R01 CA 42126-03	Human Papillomaviruses in Genital Tract Dysplasias
106.	Wettstein, Felix O. University of California (Los Angeles) 1 R01 CA 50339-01	Immunology of Virus-Induced Rabbit Papillomas/Cancer
107.	WILSON, John H. Baylor College of Medicine 5 R01 CA 15743-15	Pathways of Information Exchange in Somatic Cells
108.	YOUNG, Donald A. University of Rochester 5 R01 CA 47650-02	Papilloma Virus Actions on Host Cell Gene Products

CONTRACTS ACTIVE DURING FY89

Investigator/Institution/Contract Number	Title
109. SCHWARTZ, Dennis E. MicroProbe Corporation N44-CP-81045	DNA Probes for the Diagnosis of Human Papillomavirus Types in Man
110. SMITH, Alan E. Integrated Genetics, Inc. N44-CP-85655	Specific Antibodies to Human and Animal Polyoma Virus Tumor Antigens
111. TAUB, Floyd Digene, Limited N44-CP-85652	Identification of HPV by Non-Radioactive DNA Probes

SUMMARY REPORT

RNA VIRUS STUDIES I

The RNA Virus Studies I component of the Branch primarily involves studies of murine and primate tumor viruses and also includes projects on feline and bovine tumor viruses. In this program, extramural research is supported by several funding mechanisms: traditional research grants (R01), program project grants (P01), conference grants (R13), cooperative agreements (U01), outstanding investigator awards (R35), and first independent research support and transition (FIRST) awards (R29). The overall effort consists of 100 grants, with a funding level of 17.12 million dollars. These grants involve studies in the murine (78%), human (13%), bovine (4%) and feline (5%) model systems in the following areas: gene organization and expression, including studies of oncogenes; virus-cell interactions; characterization of the biological activity of retroviruses; studies of the inhibition of viral replication; investigations of virus-induced cell transformation; and retroviral viral vaccines.

Studies in the RNA Virus Studies I component are concerned with the elucidation of the molecular events associated with the viral conversion of normal cells to the malignant phenotype. Since the malignant phenotype is a stable inherited trait, oncogenic transformation may be the consequence of genetic alterations. This is clearly the case for cells transformed by oncogenic viruses, where specific viral genes are responsible for the initiation and maintenance of the neoplastic state. The question arises as to the function and identity of the genes responsible for naturally occurring tumors and the type(s) of genetic rearrangements thought to result in the aberrant activation of these genes. The observation that cellular homologs of viral oncogenes, in many instances, appear to be responsible for the in vitro conversion of normal cells in culture to the transformed phenotype, has spurred a search for the mechanisms by which these endogenous cellular genes may become altered to produce products with a potential for causing malignant transformation. The mechanisms responsible for the activation of cellular oncogenes may involve: local changes or mutations in genes involving base changes or small deletions which alter the functional properties of the gene product; gross changes in the relative position of genes which may either involve translocations of structural gene information from one chromosomal location to another, or the introduction of activators (such as viral long terminal repeats (LTR)) adjacent to cellular genes such that the level of gene expression is enhanced; gene amplification mechanisms which may increase the amounts of specific gene products; or changes in the activity of oncogene promoters by changing either the base sequence itself or by altering the genome structure in the vicinity of regulatory information (e.g., through changes in the pattern of methylation, the degree of supercoiling, or other aspects of chromatin structure).

Retroviruses first attracted widespread attention as oncogenic agents that replicate via DNA intermediates and involve integration of DNA copies of their genomes into the host chromosomes. No other class of animal viruses exhibits such profound intimacy with the host genome. Thus, information gathered concerning this relationship should increase our understanding of the transformation process. Retroviruses were previously classified into two groups: those that contain oncogenes and those that do not. Members of the first group (acute transforming retroviruses or rapidly transforming retroviruses) induce neoplastic

disease in infected animals within a few weeks after infection and cause rapid transformation of target cells in tissue culture. These viruses contain oncogenes (v-onc genes) that are derived from normal cellular genes, the proto-oncogenes, by recombination. Viruses of the second group (slowly transforming retroviruses) lack oncogenes, induce neoplastic disease in animals only after a long latent period (4-12 months), and do not cause transformation of tissue culture cells at a detectable frequency. Two additional types of retroviruses have been recognized. The first of these are viruses such as spleen focus-forming virus (SFFV) and mink cell focus-forming virus (MCF) which appear to be envelope gene recombinants. Although these viruses, in some cases, rapidly induce lesions in infected animals, they do not appear to carry an oncogene of the classic type (i.e., a cell-derived oncogene). Sequences located within the env region appear to be responsible for their pathogenic properties. Recently, another group of viruses without oncogenes, the lymphocyte-transforming retroviruses (T-cell lymphotropic viruses, HTLV), have been recognized. They apparently bring about cellular transformation through a novel transactivation mechanism involving a trans-acting protein encoded by the pX region (now termed tat gene) of the viral genome. Extramural research involving all four types of viruses is being administered by this component of the Biological Carcinogenesis Branch.

Virus Studies

Highlights of studies on human retroviruses can be summarized as follows. An antigenic variant of the human T-lymphotropic virus type 1 (HTLV-1) which has only rarely been isolated, is the human T-lymphotropic virus, type 2 (HTLV-2). HTLV-2 has been associated with three cases of malignancy in man, namely, two patients with unusual T-cell malignancy and a case of promyelocytic leukemia. HTLV-2 has also been isolated from rare individuals without evidence of malignancy, including a hemophiliac with unexplained pancytopenia, and an AIDS patient. Due to the low number of cases of HTLV-2 infection, it has been difficult to conclusively establish an association with, or a causative role for HTLV-2 in a specific disease in man. Currently, the differentiation between HTLV-1 and HTLV-2 is based on competition enzyme-linked immunosorbent assays (ELISA). Using this procedure, recent surveys for HTLV antibodies have suggested the prevalence of antibodies against the HTLV-2 virus in a proportion of the intravenous (IV) drug abusers in New York and in Great Britain. Because of the substantial serologic cross-reactivity between HTLV-1 and HTLV-2, it was impossible to establish conclusively that HTLV-2 infection was present among these populations. Recently, a sensitive modification of the polymerase chain reaction (PCR) method provided unambiguous molecular evidence that a significant proportion of an IV drug abuser population in New Orleans is infected with HTLV, and that a majority of these individuals are infected with HTLV-2 rather than HTLV-1. A total of 54 of the 121 samples screened were positive for HTLV (HTLV-1 or HTLV-2) by enzyme immunoassay. Thirty-three of the 54 samples were confirmed to be sero-positive by western blotting. Of 27 of these positive samples that were arbitrarily selected for further analysis, 23 were confirmed by PCR analysis to be infected with HTLV (21 with HTLV-2 and two with HTLV-1). These studies highlight and pinpoint the widespread prevalence of HTLV-2 in a proportion of an IV drug abuser population and provide a ready method for the molecular identification of a HTLV-2-infected population. The availability of this data and technology should lead to further studies to investigate the pathogenicity of HTLV-2 virus in humans (14).

Unlike many other acutely transforming retroviruses, HTLV-1, the etiologic agent of an aggressive form of human malignancy, adult T-cell leukemia, does not contain an oncogene homologous to host cellular sequences. The HTLV-1 genome encodes a 40-kD protein designated as tax1 that is not only critical for transformation, but has also been shown to transactivate the viral promoter in the long terminal repeat (LTR). Furthermore, tax1 may regulate transcription of some cellular genes, such as interleukin-2 (IL-2) and the IL-2 receptor. Recently, it was shown that HTLV-1 tax1 activates the expression of c-fos proto-oncogene in a transient coinfection assay. The endogenous fos gene expression was also increased upon transfection with a tax1 expression plasmid or by infection with HTLV-1. The proto-oncogene c-fos encodes a nuclear protein that is thought to be involved in cell growth and differentiation. An accumulating body of evidence suggests that the c-fos gene product regulates its own transcription and the transcription of several other genes. The expression of several proto-oncogenes, including the nuclear oncogenes c-fos, c-myc, and c-myb is believed to be involved in the molecular events leading to T-cell proliferation and activation. Thus the present studies suggest that transactivation of the proto-oncogene c-fos by tax1 may initiate a network of complex regulation leading to T-cell transformation (93).

The human immunodeficiency virus type 1 (HIV-1) infects cells bearing the CD4 molecule, which include certain T-cells and macrophages. HTLV-1 and HTLV-2 cause various forms of T-cell leukemia and lymphoproliferative disorders, and can immortalize peripheral blood T-cells in vitro. Since some patients are infected with HIV-1 and HTLV-1 or HTLV-2, studies were undertaken to determine whether HTLV would augment HIV production in vitro. After mitogenic stimulation by noninfectious HTLV-1 virions, peripheral blood leukocytes infected with HIV-1 in vitro produced large quantities of HIV-1. The HTLV-1 virions exerted this effect prior to, immediately following, or well after the cells were infected with HIV-1. These results provide further impetus for epidemiologic studies of dually infected individuals to determine whether HTLV-1 virions may act as a cofactor for AIDS (14).

Viruses gain entry into susceptible cells at specific cell receptor sites. Despite the wealth of information available on the life cycle of the oncogenic retroviruses, very little is known about the identities of the host cell receptors for these viruses and the purpose which the cell receptors fulfill in the life cycle of the respective host cells. The gibbon ape leukemia virus (GALV), a horizontally transmitted leukemogenic retrovirus of gibbons, productively infects human cells, but not the NIH mouse 3T3 cells. The human GALV receptor gene was molecularly cloned through appropriate transfection and cloning techniques using the mouse 3T3 cells. Upon transfection with the cloned human receptor gene, mouse 3T3 cells became susceptible to productive infection with GALV, thus demonstrating that the block to GALV replication in mouse cells is due to the absence of the cell receptor for this virus. These studies should lead to further studies to delineate the nature and functions(s) of the newly identified human cell receptor gene (55).

An important aspect of virally induced diseases is the specificity of the particular target organ or cell types affected. Many factors can influence the specificity, including both viral and cellular genes. A common viral determinant of specificity is the coat protein which determines the cell types that the virus will infect. The nondefective mouse C-type retroviruses possess similar genome structures and sequences, but they induce distinct forms of leukemia in the

mouse. To determine the genetic basis of this disease specificity, fragments were exchanged between molecular clones of a virus that induces T-cell tumors (Moloney leukemia virus) and one that induces erythroleukemias (nondefective Friend virus). It was found that the transcriptional signals in the viral LTR were the primary determinant of disease specificity. Further studies pinpointed the determinant as the enhancer region of the LTR, a sequence about 20 bases long. More detailed genetic studies showed that in recombinants between Friend and Moloney viruses, both halves of the direct repeat sequence, as well as the GC-rich segment, cooperate to establish specificity. When this portion of the enhancer is altered, the resulting virus produces a mixture of T-cell tumors and erythroleukemias. Thus, the enhancer elements confer tissue-specific gene expression on the viruses and influence the cell type which they will transform (49).

Integration of retroviruses in the host genome, a requirement for retroviral replication, can result in the induction of lethal or nonlethal mutations in the host genome, including the abnormal activation of proto-oncogenes resulting in cancer development. Such retroviral integrations at specific sites in the mouse genome have been found to cause new recessive mutations. A new mutant mouse strain, MPV17, was generated by infecting preimplantation mouse embryos with a retroviral vector. At six weeks of age, animals homozygous for the proviral insertion developed a progressive kidney disease characterized by nephrotic syndrome. All homozygous animals died of kidney failure around the age of eight weeks. When the mutated gene was cloned, it was found to be expressed in every tissue tested, but the adult kidney was the only organ where a pathological phenotype developed (53).

Mice carry 40-60 endogenous C-type retroviruses. A study was undertaken to determine the association of these proviruses with well-characterized developmental and physiologic mutations. The hairless (hr) mutation causes a variety of pleiotropic effects. Using oligonucleotide probes specific for different classes of murine leukemia virus, a provirus present in HRS/Jhr/hr mice, but absent in HRS/J+/+ mice, was identified and molecularly cloned. Genetic analyses showed perfect concordance between the phenotype and the presence of the provirus in a number of inbred and congenic strains of mice. Molecular analysis of a haired revertant established the causal relationship, since it revealed the excision of most of the proviral genome except for one long terminal repeat. These findings demonstrate that the proviral integration caused the hairless mutation and point to the utility of naturally occurring retroviral integrations for investigating the genome of the mouse (16).

Feline leukemia viruses (FeLV), the naturally occurring retroviruses of cats, are associated with a variety of proliferative and anti-proliferative diseases (e.g., leukemia, lymphoma, immunodeficiency disease, and aplastic anemia). Of the three FeLV subgroups (A, B, and C) defined thus far by viral interference and neutralization assays, FeLV of subgroup C is unique in its correlation with a specific disease, aplastic anemia. Recently, an efficient in vitro system was developed for the demonstration of the cytopathic effect of a molecularly cloned anemogenic Sarma strain of FeLV C (FSC) on erythroid progenitor cells. Forty-eight hour coculture of normal feline bone marrow mononuclear cells with an underlayer of virus-infected feline fibroblasts resulted in infection of 60% to 90% of bone marrow mononuclear cells and caused a pronounced depletion of early erythroid progenitors. The dramatic depletion of such progenitor cells was specific for this strain of FeLV and did not occur in marrow cells infected with

a molecularly cloned non-anemogenic strain of subgroup A FeLV. The ablation of erythroid progenitor cells by FSC in vitro paralleled both the decrease in the erythroid progenitor cells and the induction of aplastic anemia in vivo. Thus, the procedure of determination of both marrow cell infection by coculture and colony forming unit assessment by methylcellulose assay provides a reliable in vitro technique for studies of the mechanisms involved in retrovirus-induced marrow aplasias. In other studies, the genetic sequence responsible for the anemogenicity of the FSC strain of FeLV was localized by a molecular recombination approach. Chimeric viruses were constructed between FSC and a cloned non-anemogenic subgroup A virus and these infectious constructs, containing defined and different regions of the genomes of the respective viruses, were assayed for anemogenicity in vivo in weanling specific-pathogen-free (SPF) cats and in vitro by the colony forming assay described above. An 886 base pair region of the FSC envelope gene, which encodes the N-terminal 241 amino acids of the extracellular glycoprotein gene, was found to be responsible for the irreversible erythroid progenitor cell depletion and anemogenesis in this naturally occurring model of retrovirus-mediated bone marrow aplasia (49).

Infection by the bovine leukemia virus (BLV) is characterized by a long latent period after which some individuals develop B-cell tumors. During the latent period, the infectious virus and free virions are not found in any cells, including the lymphocytes. However, upon cultivation of the virus-infected lymphocytes in vitro, virus is produced. A longitudinal study was undertaken to determine the viral RNA detectable during this virus activation process in vitro. In situ hybridization was used to detect BLV transcripts in individual peripheral blood mononuclear cells from latently infected, asymptomatic sheep which had been previously inoculated with BLV. Peripheral blood cells that had been isolated as rapidly as possible from circulating blood, showed the presence of viral RNA only in rare cells. RNA transcripts of BLV increased in a biphasic manner within a few hours after the lymphocytes were placed in culture. Exposure to fetal bovine serum was identified as the principal cause of this transcriptional activation. Agents known to activate immune cells polyclonally caused a further increase in the number of viral RNA transcripts within eight hours after treatment. In some cases, the numbers of viral transcripts within individual cells also increased. These studies demonstrate that BLV was not detectably expressed in most resting lymphocytes circulating in the blood, but its transcription was activated by components of fetal bovine serum and can be augmented by molecules that mimic the activation of immune cells. Such activation might occur in lymphoid tissues during an immune response and may thus activate the latent viral genome, leading to the synthesis of viral regulatory proteins and tumorigenesis (74).

Using retroviral vectors, genes have been transferred with high efficiency to murine hematopoietic lineages. Therefore, retroviral vectors have been proposed as vehicles for gene therapy of heritable hematopoietic disorders. The vectors and genes transferred are stably inherited and are transmitted to cells which reconstitute the hematopoietic system of lethally irradiated mice. The application of retroviral vectors as vehicles for gene transfer to human hematopoietic progenitors has been less extensive. The utility of retroviral-mediated gene transfer in human gene therapy is dependent on the efficiency of infection by the vector, the genetic stability of the introduced genes and the level of expression of the introduced genes in the hematopoietic stem cells and hematopoietic progenitors of all lineages. Recently, the neomycin resistance gene was transferred by a retroviral vector into human hematopoietic cells. A human stromal cell line (KM101) and two human leukemic cells lines (K562 and

HL60) were used. The nontransforming gene, thus transferred by retroviral vector pZIP-SV(X), was stably and functionally expressed in primary and established lines of adherent human bone marrow stromal cells, as well as in the two human hematopoietic cell lines. To determine if differentiation of human hematopoietic cells affects the level of expression of the gene transferred, the level of neomycin-resistance gene mRNA in untreated HL60 cells was compared to that in HL60 cells which were induced to differentiate. The expression of the transferred gene increased when clones of infected HL60 cells were induced to differentiate toward the granulocytic, but not the myelocytic pathway. These results imply a correlation between the level of expression of genes transferred by retroviral vectors and the progression of hematopoietic cell differentiation. Retroviral enhancers might be involved in this regulation and such control could reflect the presence or absence of positive or negative trans-acting factor(s) (48).

Oncogene Studies

While the mechanisms of positive regulation of cell growth are increasingly well-understood, the negative regulation which limits cell proliferation remains obscure. It has become clear that the cell genome may carry a large array of genes whose function is to limit cell growth. These genes, when lost or inactivated through various mutational events, lead to tumorigenesis by removing the barrier to cell proliferation. One of the best understood examples of such suppressor genes, the Rb gene of retinoblastoma, appears to play an important role in preventing the induction of retinoblastoma, as well as other commonly occurring cancers, such as cancers of the lung and colon. Recently, evidence was obtained which advances our understanding of the molecular mechanisms of action of the Rb gene. Important preliminary evidence suggests that concomitantly with the homozygous inactivation of the Rb gene, the retinoblastoma cells also lose responsiveness to the cell-growth inhibitory substance, transforming growth factor beta (TGF-beta), due to the absence of receptors for this substance on the surface of these tumor cells. This loss of cell receptors apparently occurs as part of the pathogenic processes leading to tumor formation which may allow these tumor cells to escape the growth inhibitory influences of TGF-beta (96).

Primary rat embryo fibroblasts cannot be transformed by transfection with a single type of oncogene such as myc or ras; however, dual transfections with a nuclear oncogene, such as myc, together with a cytoplasmic oncogene, such as ras, results in the transformation of primary rat embryo fibroblasts. This demonstration of cooperation between oncogenes to cause morphological transformation of primary rat embryo fibroblasts has now been extended to a transgenic mouse system. Mid-gestation mouse embryos, which were infected with a replication-defective retroviral vector carrying the myc oncogene, developed tumors of different organs with long latent periods and at low frequencies; approximately 20% of the transgenic mice similarly inoculated with the ras oncogene developed skin tumors. However, upon simultaneous introduction of both of these oncogene-containing vectors into the genomes of mid-gestation mouse embryos, many types of tumors appeared with short latent periods, suggesting a cooperative effect between the myc and ras oncogenes. These observations suggest that a much wider range of cell types become targets for malignant transformation when the embryos are simultaneously exposed to the myc and ras oncogenes than when exposed to the same oncogenes separately. Thus, infection of mouse embryos with vectors carrying different oncogenes and oncogene combinations may be an efficient and

rapid method for evaluating the spectrum of cell types at risk for malignant conversion through cooperation between activated oncogenes (53).

Although much evidence implicates retroviral involvement in spontaneous T-cell lymphomas of high tumor incidence strains of mice, the role of retroviruses in spontaneous nonthymic lymphomas and granulocytic leukemia is not clear. A mouse B-ecotropic retrovirus isolated from inbred mouse strain BXH-2 has been found to induce predominantly myeloid leukemia in mice. Greater than 90% of this high tumor incidence strain of BXH-2 mice naturally develop myeloid leukemia by one year of age. A common site of proviral integration, presumably representing a hitherto unknown oncogene, was identified and designated Evi-2. Evi-2 maps to mouse chromosome 11. Single copy sequences of Evi-2 are present in human DNA, suggesting a human homolog of Evi-2. It appears that Evi-2 is not homologous to any of the known oncogenes, suggesting that the BXH-2-myeloid tumors may involve a set of presently unknown oncogenes. It was further found that the immunodeficient strains of mice, C57BL/6J nude and SCID (severe combined immunodeficient), are resistant to tumor induction by the BXH-2 B-ecotropic retroviruses, whereas the immunocompetent strains of mice inoculated at birth with these murine leukemia viruses develop tumors at approximately one year of age. The immunodeficient strains permit virus replication, suggesting that either the target cell is not available for tumorigenesis in vivo or that certain cellular factors, in addition to the virus, are needed for tumor formation (7).

A workshop sponsored by the RNA Virus Studies I component entitled "Human Retroviruses: Search for New Agents" was held on May 11-12, 1989. The purpose of this workshop was to assess the current state of knowledge in this field and to obtain recommendations on how the NCI could help promote research in this important area. Recommendations made by the attendees of the workshop are currently being evaluated for possible initiatives.

Thus, grants in the RNA Virus Studies I component have focused on elucidating the biology and diverse characteristics of human and other mammalian retroviruses and on their interactions with host cells resulting in the transformation of normal cells to the malignant phenotype. Recently, a polymerase chain reaction (PCR) method was developed to provide unambiguous evidence that a significant proportion of the intravenous drug abuser population in New Orleans is infected with human T-lymphotropic virus, type 2 (HTLV-2). Evidence was obtained that the trans-activation of the proto-oncogene c-fos by the regulatory protein taxl of HTLV-1 may initiate a network of complex regulation of other nuclear oncogenes, leading to the transformation of T-cells by this virus. The human cell receptor for the gibbon ape leukemia virus was molecularly cloned, and the block to replication of this virus in mouse cells was shown to be due to the absence of similar receptors in mouse cells. Evidence was obtained that HTLV virions induce the production of large quantities of the human immunodeficiency virus type-1 (HIV-1), in human peripheral blood leukocytes latently infected with HIV-1, thus suggesting that in individuals naturally dually infected with these two viruses, HTLV-1 virions may act as a cofactor for AIDS. The retroviral sequences responsible for conferring tissue tropism and type of disease induced, such as T-lymphoma and erythroleukemia in mice and aplastic anemia in cats, were defined. The adverse effect of retroviral integrations into the host genome in vivo resulting in heritable phenotypic changes, and in some cases death of the host, was demonstrated. Mechanisms of action of the suppressor Rb gene of retinoblastoma were explored and the cooperation between oncogenes in causing cancers was demonstrated in a transgenic mouse model system. A new strain of murine

leukemia virus capable of causing myeloid leukemia in mice and the common site of proviral integrations, adjacent to a new proto-oncogene in the host genome, were demonstrated. These studies thus continue to unravel the participation of viral and cellular genes in the genesis of cancer and provide insights on how the eventual control of these diseases might be achieved.

GRANTS ACTIVE DURING FY89

Investigator/Institution/Grant Number	Title
1. ARLINGHAUS, Ralph B. University of Texas System Cancer Center 5 R01 CA 45125-03	Moloney Murine Sarcoma V-mos Proteins in Cellular Transformation
2. ARLINGHAUS, Ralph B. University of Texas System Cancer Center 5 R01 CA 45217-03	A Temperature-Sensitive Retrovirus Splicing Mutant
3. AXEL, Richard Columbia University New York 5 P01 CA 23767-11	Molecular Virology
4. BALTIMORE, David Whitehead Institute for Biomedical Research 5 P01 CA 38497-05	Interactions of Oncogenes With Developing Systems
5. BARKLIS, Eric W. Oregon Health Sciences University 5 R01 CA 47088-02	Targeting of Retroviruses to Specific Cell Types
6. BARON, Samuel University of Texas Medical Branch Galveston 5 U01 CA 40764-03	Host Defenses Against HTLV-1 and 2
7. BEDIGIAN, Hendrick G. Jackson Laboratory Bar Harbor, ME 5 R01 CA 31102-09	A New Murine Model for the Study of Myeloid Leukemia
8. BESMER, Peter Sloan-Kettering Institute for Cancer Research 5 R01 CA 32926-05	C-KIT and V-KIT: Normal Function and Oncogenic Activation
9. BHARGAVA, Pushpa M. Centre for Cellular and Molecular Biology Hyderabad, India 1 R13 CA 46600-01	Symposium on Frontiers of Tomorrow in Biology

10. BOLANDER, Franklyn F., Jr.
University of South Carolina
Columbia
5 R01 CA 42009-02

MMTV Regulation in Normal Mouse Mammary Epithelium

11. BRONSON, David L.
Southwest Foundation for Biomedical Research, San Antonio
5 R01 CA 43361-03

Novel Human Retrovirus

12. BROWN, John M.
Stanford University
Palo Alto, CA
2 R01 CA 03352-33A1

Biological Aspects of Carcinogenesis by Radiation

13. CHANG, Esther H.
Henry M. Jackson Foundation
Bethesda, Maryland
5 R01 CA 45158-03

Oncogenes in Human Cancer Induction

14. CHEN, Irvin S. Y.
University of California
Los Angeles
5 R37 CA 38597-05

A Molecular Genetic Study of Human T-Cell Leukemia Virus

15. COCKERELL, Gary L.
Colorado State University
Fort Collins
1 R01 CA 43728-01

Latency and Leukemogenicity of Bovine Leukemia Virus

16. COFFIN, John M.
Tufts University
Boston
5 P01 CA 24530-10

Molecular Genetics of Cancer

17. COMPANS, Richard W.
University of Alabama
Birmingham
5 R01 CA 18611-15

Directional Transport of MuLV Glycoproteins

18. COOPER, Geoffrey M.
Dana-Farber Cancer Institute
Boston
5 R01 CA 18689-14

Infectious DNA for Endogenous RNA Tumor Virus Genes

19. CUNNINGHAM, James M.
Brigham and Women's Hospital
Boston
5 R29 CA 47075-02

Isolation and Analysis of Murine Leukemia Virus Receptor

20. DARNELL, James E., Jr.
Rockefeller University
5 P01 CA 18213-13

Correlated Program in Viral Oncology

21. DE FRANCO, Donald B. University of Pittsburgh 5 R01 CA 43037-03	Glucocorticoid Regulation of Retroviral Transcription
22. DIAMOND, Leila New York Academy of Sciences 1 R13 CA 48708-01	Viral Oncogenesis and Cell Differentiation: The Contribution of Charlotte Friend
23. DONEHOWER, Lawrence A. Baylor College of Medicine Houston 5 R01 CA 41476-03	Role of Virus and Cell Genes in Retrovirus Replication
24. DONOGHUE, Daniel J. University of California, San Diego 5 R01 CA 34456-06	Expression of Retroviral Envelope Gene Fusion Proteins
25. DUDLEY, Jacquelin P. University of Texas, Austin 5 R01 CA 34780-05	Regulation of MMTV in T-Cell Tumors
26. ELDER, John H. Scripps Clinic and Research Foundation, La Jolla, CA 5 R01 CA 25533-08	Structural Studies of Recombinant Retrovirus gp70s
27. ELDER, John H. Scripps Clinic and Research Foundation, La Jolla, CA 5 R01 CA 37830-03	Role of Recombinant Retroviruses in Murine Leukemia
28. ETKIND, Polly R. Montefiore Medical Center, NY 5 R01 CA 45583-03	Molecular Pathology of Breast Cancer
29. FAN, Hung Y. University of California Irvine 5 R01 CA 32454-09	Studies of Integrated Murine Leukemia Virus DNA
30. FAN, Hung Y. University of California Irvine 5 R01 CA 32455-09	Expression and Pathogenesis of Murine Leukemia Virus
31. FAN, Hung Y. University of California Irvine 1 R13 CA 47737-01	Workshop on Pathogenesis by Non-acute Retroviruses
32. FARAS, Anthony J. University of Minnesota 5 R01 CA 43472-02	Studies on Novel Human Endogenous Retroviruses Minneapolis

33. FLYER, David C.
Pennsylvania State University
Hershey Medical Center
5 R01 CA 44633-04

Specificity of the CTL Response to Murine Leukemia Virus

34. FOX, C. Fred
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Los Angeles
1 R13 AI 26042-01

Conference on Cell Biology of Virus Entry

35. GASPER, Peter W.
Colorado State University
Fort Collins
5 R29 CA 46371-02

Marrow Transplant Therapy for Retrovirus Infections

36. GATTONI-CELLI, Sebastiano
Massachusetts General Hospital
5 R01 CA 43499-03

Human Endogenous Retroviruses in Colon Cancer

37. GEIB, Roy W.
Indiana University School
of Medicine, Terre Haute
5 R29 CA 47944-02

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38. GIAM, Chou-Zen
University of Nebraska, Omaha
1 R01 CA 48709-01

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39. GOFF, Stephen P.
Columbia University, NY
5 R01 CA 30488-09

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40. GOFF, Stephen P.
Gordon Research Conferences
1 R13 CA 50548-01

Gordon Conference on Animal Cells and Viruses

41. GUPTA, Phalguni
University of Pittsburgh
5 U01 CA 42732-03

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42. HAAS, Martin
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San Diego
5 R01 CA 34151-08

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43. HASELTINE, William A.
Dana-Farber Cancer Institute
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5 R01 CA 36974-06

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44. HAYS, Esther F.
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5 R01 CA 12386-15

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45. HAYWARD, William S.
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for Cancer Research, New York
2 P01 CA 16599-15

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46. HAYWARD, William S.
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5 R01 CA 31491-07

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47. HINRICHS, Steven H.
University of California, Davis
1 R29 CA 49624-01

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48. HOLLAND, Christie A.
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Medical Center
Worcester
5 R01 CA 41510-04

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49. HOOVER, Edward A.
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5 R01 CA 48594-02

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50. HOPKINS, Nancy H.
Massachusetts Institute of
Technology, Boston
2 R01 CA 19308-13

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51. HUNTER, Anthony R.
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5 R35 CA 39780-05

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52. HUNTER, Eric
University of Alabama
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5 R01 CA 27834-09

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53. JAENISCH, Rudolf
Whitehead Institute for
Biomedical Research, Boston
5 R35 CA 44339-03

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54. KABAT, David
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5 R01 CA 25810-11

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55. KLINGER, Harold P.
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5 R01 CA43951-03

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56. LENZ, John R.
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5 R01 CA 44822-03

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57. LERNER, Richard A.
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5 P01 CA 27489-10

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58. LICHTMAN, Andrew H.
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5 R29 CA 43651-03

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59. LILLY, Frank
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Bronx, NY
5 R01 CA 19931-12

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60. LUFTIG, Ronald B.
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5 R01 CA 37380-07

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61. McGRATH, Charles M.
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5 R01 CA 44901-04

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62. MERUELO, Daniel
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2 R37 CA 22247-12

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63. MERUELO, Daniel
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5 R01 CA 31346-07

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64. MURPHY, Edwin C., Jr.
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5 R01 CA 34734-06

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65. OLSEN, Richard G.
Ohio State University
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2 R01 CA 40714-04

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66. PALKER, Thomas J.
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Durham, NC
2 R01 CA 40660-04

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67. PAULEY, Robert J.
Michigan Cancer Foundation
Detroit
7 R01 CA 28999-06

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68. PETERSON, David O.
Texas A and M University
College Station
5 R01 CA 32695-06

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69. PETERSON, David O.
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5 R01 CA 48041-02

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70. PHARR, Pamela N.
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1 R01 CA 50244-01

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71. PINTER, Abraham
Public Health Research Institute of the City of New York
5 R01 CA 42129-04

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72. POGO, Beatriz G. T.
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5 R01 CA 10000-21

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73. RACEVSKIS, Janis
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5 R01 CA 43864-04

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74. RADKE, Kathryn
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5 U01 CA 40653-03

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75. RADKE, Kathryn
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5 R01 CA 46374-02

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76. REDDY, Premkumar E.
The Wistar Institute of Anatomy and Biology
5 R01 CA 47937-02

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77. RISSER, Rex G.
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2 R01 CA 41302-04

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78. ROSENBERG, Naomi E.
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5 R01 CA 24220-11

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79. ROSENBERG, Naomi E.
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5 R01 CA 33771-07

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80. ROY-BURMAN, Pradip
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5 R01 CA 40590-03

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81. SARKAR, Nurul H.
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5 R01 CA 45123-02

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82. SCHWARTZ, Richard C.
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5 R29 CA 45360-03

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83. SEFTON, Bartholomew M.
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2 R01 CA 42350-04

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84. SORGE, Joseph A.
Stratagene Cloning Systems
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5 R01 CA 36448-07

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85. SRINIVAS, Ranga V.
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5 R01 CA 40440-05

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86. STEFFEN, David L.
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5 R01 CA 30674-08

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87. STEPHENS, Edward B.
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5 R29 CA 47100-03

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88. TAKETO, Makoto
Jackson Laboratory
Bar Harbor, ME
5 R01 CA 39652-03

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89. THOMAS, Christopher Y.
University of Virginia
Charlottesville
5 R01 CA 32995-07

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90. TOMPKINS, Mary B.
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5 R01 CA 43676-03

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91. VAIDYA, Akhil B.
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5 R01 CA 22413-10

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92. VAN BEVEREN, Charles P.
La Jolla Cancer Research Foundation
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5 R01 CA 42909-03

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93. VERMA, Inder M.
Salk Institute for Biological Studies, La Jolla, CA.
5 R35 CA 44360-02

Oncogenes, Proto-oncogenes and Retroviral Vectors

94. VOGT, Marguerite M.
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5 R01 CA 13608-17

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95. WACHSMAN, William
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5 R01 CA 43370-04

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96. WEINBERG, Robert A.
Whitehead Institute for Biomedical Research, Boston
5 R35 CA 39826-05

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97. WILSON, Michael C.
Scripps Clinic and Research Foundation, La Jolla, Ca.
5 R01 CA 33730-07

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98. WITTE, Owen N.
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Los Angeles
5 R01 CA 27507-10

Transformation by Abelson Murine Leukemia Virus

99. WONG, Paul K.
University of Texas M.D. Anderson Cancer Center, Smithville, TX
5 R01 CA 45124-04

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100. YOSHIMURA, Fayth K.
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Research Center, Seattle
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DNA Forms of Murine Leukemia
Viruses

SUMMARY REPORT

RNA VIRUS STUDIES II

The RNA Virus Studies II component of the Branch primarily involves studies of the avian tumor viruses and hepatitis B virus. This program consists of 91 research grants with an estimated total funding of 21.35 million dollars for FY89. Of these, approximately 83% are involved with studies of avian tumor viruses and 14% concern hepatitis B virus or other hepatitis viruses and their relationship to primary hepatocellular carcinoma. The remaining 3% deal with a variety of agents which are not as closely related to human diseases. The majority of studies funded by RNA Virus Studies II are focused on the molecular nature of the transformation process, the definition and discovery of new oncogenes (genes responsible for the transformation of cells from normal to malignant), and the development and testing of hypotheses about the mechanism(s) of oncogenesis of viruses lacking oncogenes. In addition to 65 traditional R01 grants and 4 P01 program project grants, this component now includes 9 R35 outstanding investigator awards (OIG), 4 R37 method to extend research in time (MERIT) awards as well as 1 R13 conference grant, and 8 R29 first independent research support and transition (FIRST) awards.

The elucidation of the mechanism(s) by which protein products of viral oncogenes (v-oncs) initiate and maintain the transformed state, and how their cellular counterparts (c-oncs) or proto-oncogenes acquire transforming potential, are key issues in studies of viral oncogenesis and are being actively pursued by investigators in this program component. While less is known about the functions of oncogene products than about the structure of the oncogenes themselves, knowledge of the proto-oncogene functions in the normal cell and their possible modification in the malignant cell is essential to an understanding of virus-cell interactions leading to the transformed cell phenotype. As the techniques for identifying and isolating oncogene products develop further, progress in the search for the essential transforming functions of oncogene products will also be made.

Oncogenes and Retroviruses

The src oncogene product is, perhaps, the tyrosine kinase whose structure and functional relationships are best understood. Thus it is appropriate to begin this report with selected studies on the src oncogene. The src protein product consists of three interacting domains: a carboxyl terminal kinase domain, which shares amino acid sequences with other known tyrosine kinases; a middle domain, which begins somewhere between amino acids 80-100 and extends to around amino acid 270. Mutations within this middle domain can drastically alter the ability of src genes to transform cells, but have little effect on the in vitro or in vivo tyrosine kinase activity of pp60v-src. Since transformation but not kinase activity is affected by this domain, it is believed to play a role in the interaction of the oncogene protein with cellular target proteins. It has been termed a modulatory domain, but little is known about how this domain affects substrate recognition and transformation. The sequence of the third amino terminal domain is largely unique to src. It contains the myristylation signal sequence, the site for post-translational modification by protein kinase C and cAMP-dependent protein kinases, and appears to be necessary for association with cellular membranes.

The critical cellular target proteins with which the v-src protein product must interact to generate the transformed phenotype are unknown, in part because of the low levels of these proteins in cells, and because many tyrosine phosphorylations are probably adventitious and not involved in oncogenesis. Based on immunoprecipitation studies of transformed cells, suggested possible targets have been the skeletal proteins vinculin and talin, calmodulin, the fibronectin receptor, and the EGF (epidermal growth factor) receptor. Based on gel electrophoretic separations, at least 60 proteins displayed enhanced phosphotyrosine levels in transformed cells, when compared to control cells. In normal cells, the predominant tyrosine phosphorylation was on a 42 M_r protein. The amount of phosphotyrosine on this protein was dramatically increased by stimulation of normal cells with any of a variety of mitogenic agents (89).

Since oncogenes, in general, appear to be derived from or utilize the normal mitogenic signalling machinery, and since activation of c-src to an oncogene confers a constitutive tyrosyl protein kinase activity on the protein product, it seemed likely that a protein tyrosine phosphorylation of biological relevance for transformation would also occur in a regulated fashion during normal mitogenesis. The non-receptor protein which most closely fits this description is pp42. Since the dissection of the mechanisms of oncogenic transformation will require the identification of critical cellular targets, and because of the previously described properties of pp42, the possible role of this protein in oncogenesis is being actively pursued.

A more classical approach to studying oncogene function involves the use of site-directed mutagenesis. A portion of the v-src oncogene designated src homology 2 (SH2), has been studied using this technique. This region is highly conserved among tyrosine kinases lacking trans-membrane domains. Similar amino acid sequences are also present in phospholipase C and in the new viral oncogene crk, which lacks a kinase domain, but induces tyrosine phosphorylation. Work on a particular SH2 mutant which transforms chick cells, but not rat cells, has indicated that in rat cells the mutant protein appears to induce phosphorylation of a different set of proteins than does wild-type pp60-src, but the same set of proteins is affected by both normal and mutant SH2 proteins in chicken cells. The mutant protein is somewhat less stable and has a slightly reduced specific kinase activity than wild-type protein in rat cells, but a normal amount of protein is present in the membrane fraction. The chicken gene that might be responsible for the host-dependent phenotype of this particular mutant is being actively sought. Another approach to studying oncogene function involves isolating mutants of a transformed cell line carrying functional copies of the v-src oncogene, which are no longer transformed in phenotype despite the continued expression of wild-type alleles of v-src. This cell line appears to be resistant to retransformation by several oncogenes in addition to src. The responsible mutation seems to be a dominant cellular gene. Further studies are ongoing on this interesting cell line, and attempts to isolate the mutant host gene by retroviral insertion mutagenesis could provide interesting clues to the mechanism of this resistance (83).

The kinase and transforming activities of the c-src protein are negatively regulated by phosphorylation of tyrosine 527, a residue six amino acids from its carboxyl terminus. Replacing the tyrosine at position 527 with phenylalanine has been shown to activate the c-src protein, but this activation is still less than that of the viral transforming protein. In order to determine if additional

carboxyl terminal mutations could further stimulate the transforming activity of the c-src protein, mutants were constructed in which the 17 carboxyl terminal amino acids were deleted, and in which the tyrosine closest to tyrosine 527 (tyrosine 519) was changed to phenylalanine. The change from tyrosine to phenylalanine in position 519 did not affect the activity of the c-src protein. However, the deletion of the 17 carboxyl terminal amino acids activated focus formation, growth in soft agarose, in vivo tumorigenicity and in vitro-specific kinase activity to levels intermediate between those of the native protein and the c-src protein in which the tyrosine at position 527 was changed to phenylalanine. These observations support the hypotheses that complete transformation by the c-src protein requires activation of its protein tyrosine kinase activity and the downward regulation by the c-src protein's carboxyl terminus is governed by the phosphorylation of tyrosine 527. Additional changes beyond that needed to prevent this phosphorylation do not appear to further enhance oncogenic activity (68).

Additional studies of the src proto-oncogene were carried out in tumor-derived Syrian hamster embryos (SHE) cell lines, induced in vitro by treatment with chemical carcinogens. The preneoplastic lines are morphologically transformed, immortal, and non-tumorigenic, and thus represent an intermediate population in multi-step neoplastic development. The induced cell lines contained increased levels of c-src kinase activity, compared to pre-neoplastic parental cell lines and normal SHE cells. The increased kinase activity did not result from an increase in the amount of proto-oncogene protein in the cell lines, but represented a 4 to 11-fold increase in the kinase specific activity. Both the extent of phosphorylation and the velocity of the c-src kinase phosphotransferase activity were increased in the tumor-derived cell lines. SHE cell lines producing chicken pp60c-src were isolated following cotransfection with plasmids bearing the chicken c-src and neomycin resistance genes. Chicken pp60c-src expressed in an asbestos-transformed tumor-derived cell line showed an approximate threefold activation of tyrosine kinase activity compared to chicken c-src protein expressed in the preneoplastic cell line. These results indicate that activation of the c-src protein is mediated by trans-acting cellular factors present in the tumor-derived cells. Analysis of the c-src protein in normal SHE cells, preneoplastic cell lines and tumor-derived cell lines showed no alteration in the phosphorylation of tyrosine 527 or tyrosine 416, the two tyrosine residues whose phosphorylation states have been associated with modulation of kinase activity. Thus, it appears that the neoplastic progression of cells may be accompanied by the activation of proto-oncogene products, such as the c-src tyrosine kinase, by mechanisms that may not directly involve genetic alteration of the proto-oncogene DNA sequence but instead host protein proto-oncogene interactions (59).

Another demonstration of the role of cellular factors in virally-induced transformation is derived from studies of v-rel. Transformation by the v-rel oncogene of the reticuloendotheliosis virus is primarily cell specific. While v-rel efficiently transforms chicken spleen and bone marrow stem cells in vitro and induces rapid lethal lymphomas in young birds, it does not rapidly transform chicken embryo fibroblasts. The nuclear localization of the v-rel gene product in nontransformed fibroblasts, along with its ability to function as a transforming protein in the nucleus of chicken spleen cells, suggested that the v-rel oncogene product might belong to the family of nuclear oncoproteins, and thus could express an immortalizing function in fibroblasts. To investigate the specificity of cell transformation by this oncogene, experiments were performed

to determine whether the oncogene could immortalize primary rat fibroblasts. Results of these experiments have demonstrated that, unlike other nuclear oncoproteins, the product of the v-rel oncogene did not immortalize primary rat embryo fibroblasts. However, the oncoprotein was able to cooperate in a synergistic way with the polyomavirus middle T protein in inducing efficient transformation of established rat fibroblasts by increasing the steady-state level of middle T mRNA, indicating that the v-rel protein might function as a transactivator. Cotransfection of cells from different species with the v-rel gene, along with constructs expressing the chloramphenicol acetyl transferase (CAT) gene under the control of different promoters, revealed that the v-rel oncoprotein is a cell-specific transcriptional transactivator of certain promoters. Moreover, the extent of cell-specific transactivation by v-rel correlated with toxic effect in these same cells (81).

An additional investigation of oncogene function involved a survey of the expression of 16 proto-oncogenes in chicken leukemic cells induced by the v-myb oncogene of the avian myeloblastosis virus (AMV). Of the 16 proto-oncogenes tested, five were found to be expressed (c-ets, c-fps, c-mht, c-myc, and c-rel). Four of these five proto-oncogenes, c-ets, c-mht, c-myc, and c-rel, have had part of their sequences transduced into avian retroviruses (E26, MH2, MC29, and REV-T, respectively), which are associated with hematopoietic malignancies. The fifth proto-oncogene, c-fps, is a tyrosine protein kinase whose expression is restricted to myeloid hematopoietic cells where it is involved in the control of proliferation and/or differentiation. The size of the c-fps transcript (4.0 kilobase pairs) was not in good agreement with the size (approximately 3.0 kilobase pairs) previously reported but was uniform in the leukemic cells from ten different chickens. The size of the other proto-oncogene transcripts appeared normal. Interestingly, the c-myb gene, which is the cellular progenitor of the v-myb oncogene of avian myeloblastosis virus, was not expressed in any of the leukemic cells, despite published reports that it is expressed in the immature myeloid cells which are targets for AMV transformation. This could represent down-regulation of c-myb by v-myb, or a differentiation-related arrest of c-myb expression. The leukemic phenotype induced by v-myb may therefore become expressed at a later stage of myeloid differentiation when c-myb expression is repressed (3).

The final study on oncogenes in this report deals with the erb-B oncogene. Three new erb-B transducing viruses generated during avian leukosis virus-induced erythroblastosis have been cloned and sequenced, and their transforming abilities analyzed. One provirus designated E1 expresses an amino-terminally truncated erb-B product that is analogous to the proviral insertionally activated c-erb-B gag-erb-B fusion product. This virus efficiently induces erythroblastosis, but does not transform fibroblasts in vitro or induce sarcomas in vivo. In contrast, another virus designated S3 expresses an erb-B product similar to the first erb-B product, but having a large internal deletion located between the kinase domain and the putative autophosphorylation site, P1. Interestingly, this virus is no longer capable of inducing erythroblastosis, but can induce both fibrosarcomas and hemangiomas in vivo. Still another provirus (F3) has sustained an approximately 23-amino-acid carboxyl terminal truncation and is capable of inducing both erythroblastosis and sarcomas. This virus expresses an erb-B product with the shortest carboxyl terminal region sufficient to maintain the sarcomagenic potential of the protein. The distinct transforming properties of these viruses indicate that the different structural domains of the erb-B product confer distinct disease specificities (44,53).

A major difficulty in producing proteins on a large scale in mammalian cells has been the contamination of the desired protein by large amounts of serum proteins used in the growth medium. Retroviral vectors have been produced in which the gag gene is linked to the protein of interest. This technique results in the production of fusion proteins which migrate to the cell surface. Interaction of the gag portion of the molecules with one another results in budding, in which the fusion proteins become membrane enclosed and other cytoplasmic proteins are excluded. As the budding process nears completion, the protein of interest is cleaved from the retrovirus protein by a cellular protease associated with the site of budding. The enveloped particles are then released into the growth medium, and can be purified by centrifugation in the same way that a retrovirus can be purified. Since only one of the retrovirus genes is used in the process, the particles are not infectious, are safe to handle, and offer a convenient means of large-scale protein production in mammalian cell systems. This technique, called "retro-secretion," is currently being patented (91).

Efforts continue to elucidate the mechanisms of oncogenesis by both rapidly and slowly transforming oncogenic retroviruses; and to achieve these ends investigators are attempting to identify critical cellular targets of the viral oncogenes, to identify genes which are involved in a resistance to virally induced oncogenic transformation and to determine how changes either in the viral genome or in viral proteins can result in altered tissue and/or disease specificity.

Hepadnaviruses

The other major group of studies supported by this component involves the hepatitis B virus (HBV) which chronically infects 300 million people worldwide. Without some intervention strategy, all of these chronic carriers will die of primary hepatocellular carcinoma unless they first die of some other unrelated cause. Thus, studies on the hepatitis viruses, their interaction with human and animal hosts, and possible mechanisms of hepatocarcinogenesis are matters of high priority.

Hepatitis B virus DNA has been found to be integrated into the cellular genome of hepatocellular carcinoma (HCC) tissue in hepatitis B surface antigen (HBsAg) carriers from all areas of the world. Integration of HBV DNA has also been found in the genome of liver cells of long-term viral carriers without liver cancer, and is thought to precede hepatic oncogenesis by months or years. In areas where HBV carrier rates and HCC incidence are high, initial infection occurs during infancy or early childhood. The level of viremia generally decreases over time, and a significant portion of carriers convert spontaneously from hepatitis B-e antigen (HBeAg) to anti-HBeAg status associated with cessation of virus production. On the average, HBV carriers with HCC show lower levels of viremia than HBV carriers without HCC, and most virus carriers convert to anti-HBeAg status before malignancy develops. In black South Africans with HCC, reduced levels or the absence of viremia has been observed, even when such patients remain HBeAg-positive. However, molecular events leading to reduction (or suppression) of HBV replication in long-term carriers and the precise role of viral DNA integration in the development of hepatic malignancy have not yet been elucidated. Full-length free HBV genomes and lower molecular weight viral DNA forms (replicative intermediates) are present in the liver of HBeAg-positive carriers with active virus replication, whereas only integrated HBV DNA or integrated forms plus free genomes, but no replicative intermediates, are present in the liver of carriers

who no longer shed measurable quantities of virus into the serum. At the time of HBeAg/anti-HBeAg, viral carriers show loss of HBV replicative forms but retention of free viral genomes.

To understand the molecular events in the progression from replicative to non-replicative infection, the molecular forms of HBV DNA present in liver and tumor tissue, the expression of viral surface and core proteins, and virus levels in the serum of 13 black South African HBV carriers with HCC were studied. A stage during persistent infection was identified in which viral DNA replication continues in the liver, with accumulation of replicative forms in the cytoplasm, but in which virus secretion is reduced or absent. Surface antigen synthesis continues normally, but little or no core antigen can be detected immunologically in liver tissue of these patients. This suggests a block in virus assembly or secretion, which is either secondary to reduced core antigen synthesis or is the result of production of a modified or defective core antigen, which precludes normal virus particle formation. This stage, in which HPV DNA replication occurs without virus secretion, may represent a critical period during progression from replicative to non-replicative HBV infection. At this stage, there is an accumulation in the hepatocyte of extrachromosomal viral DNA, a substantial portion of which might not be sequestered in virions or normal viral core particles. Although this viral DNA is separated from the cellular genome by the nuclear membrane, such boundaries are temporarily lost during mitosis, which is increased during liver inflammation and regeneration. Therefore, aberrant replicative forms of HBV DNA may serve as a substrate for integration of viral DNA sequences into the host genome. Genetic changes caused by these integrations may play a role in oncogenic transformation, since these integrations are clonally selected during tumorigenesis (67).

Other studies on HBV involved transgenic mice and were designed to test the hypothesis that the HBV enhancer directs liver-specific gene expression. A fusion gene containing the HBV enhancer, linked to the SV40 early promoter and T antigen gene, was constructed and introduced into the germ line of transgenic mice. Of the two transgenic mice derived, one had multiple copies of the transgene in two unique integration sites. At 7-1/2 weeks of age, this animal was sacrificed and necropsy revealed a tumor at the base of the skull involving the pituitary and trigeminal nerve, and a choroid plexus tumor in both lateral ventricles and the fourth ventricle. Cell lines established from the choroid plexus tumor were positive for T antigen expression, as assayed by immunohistochemistry. Northern analysis revealed the presence of T antigen mRNA in the tumor-derived cell line of this animal, but not in the liver. With the exception of bilateral cataracts, the second transgenic mouse appeared normal at the same age. Necropsy at 22 weeks revealed a tumor at the base of the brain and tumors in both ovaries, as well as cystic kidneys. In both animals the liver appeared histologically normal. These data indicate that the HBV enhancer can stimulate expression of a heterologous gene in vivo in a variety of different tissues (13).

In other animal studies, hepatocellular carcinomas induced in two woodchucks chronically infected with the woodchuck hepatitis virus were characterized for viral integration near c-myc and for alterations of c-myc expression. In one tumor, viral integration within the untranslated region of c-myc exon 3 resulted in overexpression of a long c-myc viral cotranscript. In the second tumor, a single insertion of a highly rearranged viral sequence 600 base pairs upstream of the c-myc exon 1 was associated with increased levels of normal c-myc mRNA. In

both cases, viral enhancer insertion and disruption of normal c-myc transcriptional or post-transcriptional control appeared to be involved in c-myc activation. These results from the laboratory of Dr. Pierre Tiollais at the Pasteur Institute demonstrate that integration of woodchuck hepatitis virus near a cellular proto-oncogene, as in several retroviral models, can contribute to the genesis of liver tumors.

An interesting but unresolved issue is the function of the so-called X gene. The sequences of the mammalian hepadnaviruses contain four overlapping long open-reading frames. Three of these coding regions have been assigned to known viral proteins: the preS/S region encodes the viral surface protein, the C region encodes the structural protein of the nucleocapsid, and the P open-reading frame encodes the viral polymerase. The product of the fourth open-reading frame, X, displays no convincing homology with known protein sequences. It has not been successfully purified from virus-infected cells, and has proven difficult to analyze immunocytochemically. Further, while the first study of an X gene deletion mutant reported a wild-type phenotype for virus replication in cultured cells, the length and conservation of the open-reading frame, the appearance of anti-X antibodies in the course of natural infection, and the failure of X frameshift mutants to grow in animal hosts, all argue strongly that the X protein plays an important role in the virus life cycle.

A computer analysis of the sequence conservation, hydrophobicity, and potential secondary structures of the predicted X polypeptide, point to a small, soluble, intracellular protein without a signal sequence or other obvious identifying sequence motifs. Comparison of this data with other viral families suggests a hypothesis that the X protein might be a transactivator of gene expression. Experiments by several groups showed that X expression can increase the expression in trans of reporter genes in a variety of heterologous contexts. In these experiments, plasmids bearing chloramphenicol acetyl transferase (CAT) genes expressed from any of several nonhepadnaviral promoters or HBV enhancer and core promoter were cotransfected into several non-permissive cell lines along with plasmids bearing the HBV X gene. Transactivation of the CAT genes in an X-dependent fashion was observed. These studies clearly show a transactivating effect of the X gene product and are consistent with the hypothesis that transactivation of hepadnaviral promoters may be the natural function of the X gene product. However, several factors limit the inferences that can be drawn from these experiments. The cells used in these studies were not permissive for virus replication; hence some cellular factors important for virus growth, and with which the X product might interact, are absent. Since no virus replication was ongoing, other viral factors that could affect the structure or function of the protein were also absent. Finally, in the absence of firm knowledge of the structure of the true X message RNA and its protein product in vivo, recombinant X expression vectors might not produce the authentic structural form of the molecule. Many of these issues can now be directly addressed, since cell lines which support hepadnaviral replication have recently become available. Accordingly, the X-dependent transactivation was reexamined in permissive cells using hepadnaviral sequences as both the source of and the target for X activity. The results of these experiments demonstrated that transactivation by the hepadnaviral X gene product does indeed occur during authentic virus replication, and that the X products of all mammalian hepadnaviruses possess transactivating activity. These findings suggest that the effects of the X gene product are most likely exerted through a ubiquitous and sequence non-specific component of the

gene expression machinery, rather than through a sequence-specific DNA binding event (82).

During this reporting period, this program component and the Office of the Branch Chief participated, both as members of the planning committee and as participants, in an international workshop on pathogenesis and prevention of hepatocellular carcinoma, co-sponsored by the Fogarty International Center and the NCI. Currently, the results of that meeting are being reviewed in terms of initiatives which could be supported through a request for grant applications. Discussions have also been held with the Fogarty International Center regarding possible co-funding of foreign aspects of some of these studies. An additional workshop is being planned in the area of oncogene control of the cell cycle. This workshop, built on a solid background of experimental studies funded through the Biological Carcinogenesis Program, will not only furnish a state-of-the-art description of the possibilities of oncogene-induced cell cycle control as a mechanism for oncogenesis, but may also suggest fruitful areas for further program initiatives.

In summary, the search for new viral onc genes and for their mechanism(s) of action may uncover interesting new genes that are components of the growth regulatory system of the cell. The knowledge gained through basic studies of retroviral proteins has provided the basis for new techniques for protein production and purification in mammalian cells which may be useful in production of vaccines or other biologically active compounds. Studies of the hepadnaviruses are beginning to suggest mechanisms of hepatocarcinogenesis; and the use of the new transgene technology should add substantially to our ability to determine the targets of various oncogenes, their mechanisms of action, and perhaps suggest means of interfering with or interrupting the malignant process.

RNA VIRUS STUDIES II

GRANTS ACTIVE DURING FY89

Investigator/Institution/Grant Number	Title
1. ACS, George Mount Sinai School of Medicine 5 R01 CA 34818-05	Studies on the Replication and Oncogenicity of HBV
2. BALDUZZI, Piero C. University of Rochester 5 R01 CA 32310-07	The Transforming Genes of Avian RNA Tumor Viruses
3. BALUDA, Marcel A. University of California (Los Angeles) 2 R01 CA 10197-22	Tumor Induction by Avian Myeloblastosis Virus
4. BEEMON, Karen L. Johns Hopkins University 5 R01 CA 33199-07	Location and Function of M6A in Retrovirus RNAs
5. BEEMON, Karen L. Johns Hopkins University 1 R01 CA 48746-01	Retroviral Regulatory Sequences Within Coding Sequences
6. BISHOP, J. Michael University of California (San Francisco) 5 R35 CA 44338-03	Retroviruses and Cancer Genes
7. BOETTIGER, David E. University of Pennsylvania 1 R01 CA 49866-01	Role of Integrin in Viral Transformation
8. BOETTIGER, David E. University of Pennsylvania 5 R01 CA 30383-09	Virus-Induced Myeloid Leukemia
9. BOETTIGER, David E. University of Pennsylvania 5 R01 CA 16502-15	Genetic Analysis of RNA Tumor Viruses
10. BOS, Timothy J. Eastern Virginia Medical School (Norfolk) 1 R29 CA 51982-01	Study of Target Genes Activated by the *jun* Oncoprotein
11. BOSE, Henry R., Jr. University of Texas at Austin 5 R01 CA 33192-06	Transformation by Avian Reticuloendotheliosis Virus

12. BRUGGE, Joan S.
University of Pennsylvania
7 R37 CA 27951-10
Regulation of pp60-src and the Polyoma mT Protein Interaction

13. BURK, Robert D.
Albert Einstein College of Medicine of Yeshiva University
5 R01 CA 45476-03
Role of Hepatitis B Virus in Hepatocellular Carcinoma

14. BURR, John G.
University of Texas at Dallas
1 R29 CA 47098-01
Purification of Tyrosine Phosphoproteins

15. BUSS, Janice E.
La Jolla Cancer Research
5 R29 CA 42348-04
Attachment of Myristic Acid to p60-src

16. BUTEL, Janet S.
Baylor College of Medicine
5 R01 CA 37257-03
Hepatitis B Virus and Human Liver Cancer

17. CARBON, John A.
University of California (Santa Barbara)
5 R01 CA 11034-21
Studies on Centromere Structure and Function

18. CASPAR, Donald L.
Brandeis University
5 R01 CA 15468-15
Assembly of Viruses, Membranes, and Tissue

19. CASPAR, Donald L.
Brandeis University
5 R35 CA 47439-02
Switching in Virus and Membrane Assemblies

20. CHEN, Ji Hshiung
University of Texas (M. D. Anderson Cancer Center)
5 R01 CA 42859-04
Transforming Gene of Avian Acute Leukemia Virus E26

21. CHISARI, Francis V.
Scripps Clinic and Research Foundation
5 R01 CA 40489-05
Pathogenesis of Hepatitis B

22. COFFIN, John M.
Tufts University
5 R35 CA 44385-03
Molecular Biology of Retroviruses

23. COOPER, Jonathan A.
Fred Hutchinson Cancer Research Center
5 R01 CA 41072-03
Protein Phosphorylation and Cell Growth Regulation

24.	DUESBERG, Peter H. University of California (Berkeley) 5 R35 CA 39915-05	Retroviral onc Genes and Cellular Proto-onc Genes
25.	EISENMAN, Robert N. Fred Hutchinson Cancer Research Center 5 R01 CA 20525-13	Control Mechanisms in Avian Oncornavirus Replication
26.	FARAS, Anthony J. University of Minnesota 2 R01 CA 18303-14	RNA-Directed DNA Polymerase and 70S RNA of Oncornaviruses
27.	FEITELSON, Mark A. Fox Chase Cancer Center 5 R29 CA 48656-02	Products of the X and Polymerase Genes of Hepadnaviruses
28.	GANEM, Donald E. University of California (San Francisco) 1 R13 AI 26936-01	Hepatitis B Virus Meeting
29.	GILMORE, Thomas D. Boston University 5 R29 CA 47763-02	Transformation of Cells by the v-rel Oncogene
30.	GOLDBERG, Allan R. The Rockefeller University 5 R01 CA 13362-17	Rous Sarcoma Virus Functions Involved in Transformation
31.	GOULIAN, Mehran University of California (San Diego) 5 R01 CA 11705-20	DNA Synthesis Studies
32.	GRANDGENETT, Duane P. St. Louis University Medical Center 5 R01 CA 16312-16	Avian Retrovirus DNA Synthesis and Integration
33.	GRANOFF, Allan St. Jude Children's Research Hospital 5 R01 CA 07055-26	Lucke Tumor-Associated Viruses
34.	HALPERN, Michael S. Wistar Institute of Anatomy and Biology 5 R01 CA 31514-08	Endogenous Retrovirus as a Determinant of Tumor Immunity
35.	HANAFUSA, Hidesaburo Rockefeller University 5 R35 CA 44356-03	Analysis of Cell Transformation by Retrovirus

36. HAYWARD, William S.
Sloan-Kettering Institute for Cancer Research
5 R01 CA 43250-04
Mechanisms of Viral and Non-Viral Oncogenesis

37. HUMPHRIES, Eric H.
University of Texas Health Science Center (Dallas)
5 R01 CA 32295-07
Characterization of the Avian Leukosis Virus-Induced Transformed Follicle

38. HUMPHRIES, Eric H.
University of Texas Health Science Center (Dallas)
2 R01 CA 41450-04
Expression and Function of v-rel in Lymphoid Tissue

39. HUNTER, Eric
University of Alabama (Birmingham)
2 R37 CA 29884-09
Site-Specific Mutagenesis in the Envelope Gene of Rous Sarcoma Virus

40. JOKLIK, Wolfgang K.
Duke University
5 P01 CA 30246-09
Regulatory Functions of Protein Nucleic Acid Interaction

41. JOVE, Richard
University of Michigan
5 R29 CA 47809-02
Mechanisms of Cell Transformation by the Viral src Gene

42. KNOWLES, Barbara B.
Wistar Institute of Anatomy and Biology
5 R01 CA 37225-05
Hepatitis Virus and Primary Hepatocellular Carcinoma Cells

43. KOPROWSKI, Hilary
Wistar Institute of Anatomy and Biology
5 P01 CA 21124-12
Virology and Genetics of Cancer

44. KUNG, Hsing-Jien
Case Western Reserve University
5 R01 CA 39207-05
Avian Erythroleukemia and c-erb-B Activation

45. KUNG, Hsing-Jien
Case Western Reserve University
5 R01 CA 46613-02
Oncogene Activation in Avian B and T Lymphoma

46. LEIS, Jonathan P.
Case Western Reserve University
2 R01 CA 38046-06
Retroviral Proteins Involved in DNA Integration/Virion

47. LINIAL, Maxine L.
Fred Hutchinson Cancer Research Center
5 R01 CA 18282-14
Retroviral Coded Functions

48. LIPSICK, Joseph S.
University of California (San Diego)
5 R01 CA 43592-03
Mechanism of Transformation by the v-myb Oncogene

49. MACARA, Ian G.
University of Rochester Medical Center
5 R01 CA 38888-05
Oncogenes and Control of Phosphoinositide Cycle/Kinase C

50. MAJORS, John E.
Washington University School of Medicine
5 R01 CA 38994-05
Analysis of Retroviral Transcriptional Regulation

51. MARTIN, G. Steven
University of California (Berkeley)
5 R01 CA 17542-14
Genetics of RNA Tumor Viruses

52. MILLER, Arthur D.
Fred Hutchinson Cancer Research Center
5 R01 CA 41455-03
Gene Transfer Using Retroviral Vectors

53. MOSCOVICI, Carlo
University of Florida
5 R01 CA 10697-22
Avian Leukemia Viruses and Cell Differentiation

54. NEIMAN, Paul E.
Fred Hutchinson Cancer Research Center
5 P01 CA 28151-10
Retroviruses and Cancer

55. NEIMAN, Paul E.
Fred Hutchinson Cancer Research Center
5 R01 CA 20068-14
Molecular Mechanisms in Neoplasia

56. OZTURK, Mehmet
Massachusetts General Hospital
1 R29 CA 49832-01
Studies on Human Hepatoma Cell Surface Protein p50

57. PARSONS, J. Thomas
University of Virginia (Charlottesville)
5 R01 CA 27578-09
Expression of Avian Retrovirus Transforming Genes

58. PARSONS, J. Thomas
University of Virginia (Charlottesville)
5 R01 CA 29243-09
Avian Sarcoma Virus-Specific Tumor Antigens

59. PARSONS, Sarah J.
University of Virginia
(Charlottesville)
5 R01 CA 39438-05

Role of C-src in Retroviral Transformation

60. PERDUE, Michael L.
U. S. Agricultural Research Service
(Athens, GA)
5 R01 CA 45134-03

Regulation of Protein Synthesis by the Retrovirus Leader

61. PRIVALSKY, Martin L.
University of California
(Davis)
5 R01 CA 38823-05

Characterization of v-erb-B Oncogene Protein of AEV

62. ROBINSON, Harriet L.
University of Massachusetts
Medical School
5 R01 CA 27223-10

Avian Leukosis Viruses and Cancer

63. ROBINSON, Harriet L.
University of Massachusetts
Medical School
5 R01 CA 23086-12

Retrovirus-Host Interactions

64. ROGLER, Charles E.
Albert Einstein College of Medicine
of Yeshiva University
5 R01 CA 37232-06

WHV- and HBV-Associated Hepatocellular Carcinoma

65. ROHRSCHNEIDER, Larry R.
Fred Hutchinson Cancer Research
Center
2 R01 CA 20551-13

Mechanisms of Oncornavirus-Induced Transformation

66. SEFTON, Bartholomew M.
Salk Institute for Biological
Studies
5 R01 CA 17289-14

Membranes and Viral Transformation

67. SHAFRITZ, David A.
Albert Einstein College of
Medicine of Yeshiva University
5 R01 CA 32605-08

Hepatitis B Virus - Chronic Hepatitis - Liver Cancer

68. SHALLOWAY, David I.
Pennsylvania State University
(University Park)
5 R01 CA 32317-08

Role of pp60C-src Homolog of the RSV Oncogenic Protein

69. SHENK, Thomas E.
Princeton University
2 R01 CA 39606-04

Functional Analysis of the Adeno-Associated Virus Genome

70. SHIH, Chiaho
University of Pennsylvania
5 ROl CA 43835-03
Integration of Hepatitis B Virus and Liver Neoplasia

71. SHIH, Chiaho
University of Pennsylvania
1 ROl CA 48198-01
Dissection of the Life Cycle of Human Hepatitis B Virus

72. SIDDIQUI, Aleem
University of Colorado Health Sciences Center
5 ROl CA 33135-06
Expression of Hepatitis B Virus Genes and Hepatoma

73. SKALKA, Anna M.
Institute for Cancer Research (Philadelphia)
1 ROl CA 48703-01
Retroviral RNA and Protein Processing

74. SKALKA, Anna M.
Institute for Cancer Research (Philadelphia)
1 ROl CA 49042-01
RNA Tumor Viruses: DNA Synthesis and Integration

75. SMITH, Ralph E.
Colorado State University
5 ROl CA 35984-07
Biochemistry of RNA Tumor Virus Replication

76. STAVNEZER, Edward
University of Cincinnati
2 ROl CA 43600-04
Origin, Structure and Biological Activity of SKVS

77. STOLTZFUS, Conrad M.
University of Iowa
5 ROl CA 28051-10
Avian Retrovirus RNA Metabolism

78. SUMMERS, Jesse W.
University of New Mexico
5 R35 CA 42542-04
Persistent Infections by Hepadnaviruses

79. SWANSTROM, Ronald I.
University of North Carolina (Chapel Hill)
5 ROl CA 33147-06
Retrovirus Replication: Interaction with the Host Genome

80. TATTERSALL, Peter J.
Yale University School of Medicine
5 ROl CA 29303-09
Molecular Basis of Parvovirus Target Cell Specificity

81. TEMIN, Howard M.
University of Wisconsin (Madison)
5 POl CA 22443-12
Molecular Biology and Genetics of Tumor Viruses

82. VARMUS, Harold E.
University of California
(San Francisco)
5 R01 CA 37281-06

Oncogenic Potential of the Hepatitis B-Type Viruses

83. VARMUS, Harold E.
University of California
(San Francisco)
5 R35 CA 39832-05

Molecular Analysis of Retroviruses and Oncogenes

84. VOGT, Peter K.
University of Southern California
5 R35 CA 42564-04

Onc Genes in Virus and Cell

85. VOGT, Volker M.
Cornell University (Ithaca)
5 R37 CA 20081-13

Avian Retrovirus Structure and Assembly

86. WANDS, Jack R.
Massachusetts General Hospital
5 R01 CA 35711-06

Pathogenesis, Immunodiagnosis, and Therapy of Carcinoma

87. WANG, Lu-Hai
Mount Sinai School of Medicine
5 R01 CA 29339-10

Transforming Genes of Avian Sarcoma Viruses

88. WANG, Lu-Hai
Mount Sinai School of Medicine
5 R01 CA 49400-02

Expression and Function of Proto-oncogene C-src

89. WEBER, Michael J.
University of Virginia
(Charlottesville)
2 R37 CA 39076-06

Signal Transmission by the src Oncogene

90. WEINTRAUB, Harold M.
Fred Hutchinson Cancer
Research Center
5 R35 CA 42506-04

Generation of Development Mutants with Cloned DNA Vectors

91. WILLS, John W.
Louisiana State University
Medical School (Shreveport)
5 R29 CA 47482-03

Analysis of Retrovirus Assembly by In Vitro Mutagenesis

SUMMARY REPORT

AIDS VIRUS STUDIES

The AIDS Virus Studies component supports research on the human immunodeficiency virus (HIV) and other related retroviruses as models of HIV infection and its associated neoplastic sequelae. Currently, 24 research grants are in the program with an estimated funding of 4.86 million dollars, including 20 traditional research grants, 1 outstanding investigator award (OIG), 1 method to extend research in time (MERIT) award, 1 first independent research support and transition (FIRST) award and 1 conference grant. In addition, there are 2 Phase I small business innovative research (SBIR) contracts. Current research supported by the AIDS Virus Studies component focuses on development of animal models for basic research in HIV and lentivirus pathogenesis (43%); the etiologic role of HIV in AIDS and other diseases (22%); molecular mechanisms of viral replication and gene expression (13%); basic laboratory research for the development of experimental vaccines (10%); the association of HIV with an increased incidence of neoplastic sequelae (8%); and viral mechanisms involved in immunopathogenesis, immune system dysfunction and lymphocyte depletion (4%).

AIDS is characterized by lymphopenia with a marked reduction in the numbers of T4 helper cells (lymphocytes) and suppression of immune system functions leading to an increase in opportunistic infections and malignant sequelae. Scientists and clinicians now know that the control of HIV infections and AIDS will be difficult due to the broad tissue tropism of the virus, its ability to evade the immune system upon infection and the virus' ability to establish latent infections. Difficulties encountered in the design and evaluation of experimental vaccines and the recent emergence of drug-resistant strains of HIV have serious implications for treating the disease and preventing its spread. In the initial stage of HIV infection, the envelope glycoprotein (gp120) interacts with the CD4 lymphocyte surface antigen, followed by the introduction of the viral RNA genome into the cell's cytoplasm. HIV infects cells expressing CD4 antigen, including T- and B-lymphocytes, monocytes and macrophages, and some cells of neuronal or glial origin. Later in the infectious process, the virus induces a decrease in the number of circulating $CD4^+$ lymphocytes, leading to clinical symptoms of immunodeficiency and disease progression.

Researchers have observed a differential cytopathogenicity of HIV clinical isolates. In vitro studies carried out by Dr. David Volsky demonstrated that several HIV isolates from a single recombinant DNA clone of HIV-1 possess markedly different biological properties in terms of their ability to replicate and induce cytolysis in a variety of host cells. A cytopathic HIV-1 isolate containing multiple virus genotypes was molecularly cloned, and the biological activity of randomly selected clones was assessed by transfection into human lymphoid or glial cell lines. Five infectious clones were distinguished from one another by restriction endonuclease mapping. Each clone exhibited a distinct host cell range as well as markedly different infection kinetics and cytopathic properties when tested in human cell lines of T-lymphocytic, monocytic, and astrocytic origin. In particular, infection with one HIV-1 clone, NIT-E, was characterized by slow growth kinetics and lack of significant cytopathic effects in acutely and chronically infected cells. In contrast, another clone, NIT-A, exhibited a wide host cell range, fast kinetics of infection and a high degree of cytopathogenicity and thus was similar to the parental isolate. These data indicate that individuals infected with HIV may carry multiple HIV-1 genotypes

with distinct cytopathic potentials and cell tropisms. Thus, in assessing therapeutic efficacy of drugs or prophylactic abilities of vaccines, it is important for investigators to recognize that they may be analyzing a mixed population of viruses (24).

Two possible mechanisms have been proposed for the entry of enveloped viruses into cells: the direct fusion of the viral envelope with the plasma membrane or internalization of the virus-receptor complex by receptor-mediated endocytosis. The observation that CD4 on T-cells is internalized suggests that entry of HIV may proceed via receptor-mediated endocytosis. Dr. Richard Axel has developed a tissue culture system permitting genetic experiments to examine the role of CD4 receptor-mediated endocytosis of HIV. Recombinant or mutant DNA copies of the CD4 gene were introduced into HeLa cells. $CD4^+$ HeLa transformants (those expressing the normal CD4 protein) are susceptible to viral infection; however, HeLa cells expressing mutant CD4 molecules defective in endocytosis also remained susceptible to HIV infection. While viral entry was equally efficient in cells bearing wild-type or mutant CD4 molecules, the binding of HIV to CD4 on the cell surface did not result in endocytosis of the CD4 molecule. From these observations, Dr. Axel hypothesized a model of infection in which HIV initially associates with the CD4 receptor and then introduces its genomic RNA into the cell through direct fusion of the viral envelope with the plasma membrane. Unlike infection by most retroviruses, infection by HIV is cytopathic, a property that may be a consequence of the unique ability of the viral envelope glycoprotein to fuse directly with the cell membrane. These observations provide information that may be useful in designing prophylactic agents to prevent the spread of HIV from cell to cell (RNA I component, reference 3).

Direct fusion of cells infected with HIV, mediated by the gp120 envelope glycoprotein and the gp41 transmembrane protein, is thought to cause the depletion of $CD4^+$ lymphocytes associated with AIDS disease progression. Based on his previous investigations with ecotropic murine leukemia viruses (MuLV) which demonstrated the essential role of efficient envelope protein processing in retrovirus-induced cell fusion, Dr. Rex Risser developed an analogous in vitro system to study HIV env cell fusion. Site-directed mutagenesis has been carried out on the HIV env sequences encoding the proteolytic cleavage site for the processing of gp160 precursor protein into the gp120 and gp41 envelope proteins, and the sequences encoding the amino terminal hydrophobic domain of gp41. Dr. Risser's results demonstrate that highly conserved regions of the envelope protein play essential roles in HIV envelope-induced cell fusion. Inhibition of HIV envelope protein processing provides a means of preventing HIV-induced depletion of $CD4^+$ lymphocytes (RNA II component, reference 81).

In order to study the structural basis of the association of CD4 with the HIV envelope glycoprotein, Dr. Richard Axel has prepared a recombinant soluble form of the HIV cellular receptor (rsCD4). CD4 exhibits an immunoglobulin-like structure with four tandem extracellular domains which share homology with immunoglobulin light chains. Dr. Axel has carried out analysis of site-specific CD4 mutants to more precisely determine the site of association of the CD4 molecule with HIV. These studies indicate that a short contiguous region of eight amino acids in the amino terminal portion of CD4 may constitute the contact regions responsible for the high affinity binding of HIV gp120 to CD4. Dr. Axel's experiments provide convincing evidence that this CD4 domain is likely to interact with a cavity in the envelope of the virus independent of other structural features of CD4. From the size of the interacting region of CD4,

Dr. Axel estimates that the size of the envelope cavity is likely to be small relative to the size of an average humoral antibody. The implication of these observations is that the AIDS virus appears to have evolved a receptor recognition mechanism which may be inaccessible to the humoral immune response (RNA I component, reference 3).

The simian immunodeficiency virus (SIV) induces an AIDS-like disease in rhesus monkeys, with clinical symptoms and disease progression similar to those observed in AIDS patients, providing an appropriate model for investigating pathogenic mechanism and for the evaluation of therapeutic agents for treating AIDS. Dr. Norman Letvin has demonstrated that recombinant soluble CD4 (rsCD4) is a potent inhibitor of HIV and SIV replication in vitro. To assess the therapeutic efficacy of rsCD4 in preventing SIV disease progression, SIV-infected monkeys received daily intramuscular injections of rsCD4. While SIV was readily isolated from peripheral blood lymphocytes and bone marrow cells of these animals prior to treatment, SIV became more difficult to isolate soon after treatment with rsCD4 had begun and continued to be so throughout the treatment period; Dr. Letvin hypothesizes that this may reflect a quantitative decrease in replication of SIV in the treated animals. In addition, the ability to grow granulocyte-macrophage (GM-CFU) and erythrocyte progenitor colonies from the monkeys, which was diminished by SIV infection, was restored to normal levels during the treatment period. However, within 60 to 90 days after treatment was discontinued, SIV could once again be isolated from the animals and the ability to grow GM-CFU was reduced. It was thought that rsCD4 could act by several mechanisms: by absorbing soluble SIV envelope glycoprotein and thus interfering with cytolytic T lymphocytes from killing virus-infected lymphocytes; by inhibiting the fusion of virus-infected cells with uninfected cells, thus inhibiting the spread of virus; or by directly blocking the interaction of virus with CD4 molecules on target T lymphocytes. Thus, rsCD4 treatment could have beneficial therapeutic value in the treatment of AIDS patients (6).

The design of therapeutic agents to inhibit HIV replication requires a thorough knowledge of the functions of HIV structural and regulatory proteins. One such regulatory protein, tat, is a 14-kilodalton (kD) nuclear protein that acts in trans to stimulate HIV gene expression; in addition, expression of tat is required for viral replication. Both transcriptional and post-transcriptional mechanisms have been proposed as mechanisms through which tat regulates HIV gene expression. Using site-specific mutagenesis techniques, Dr. William Haseltine identified three potential functional domains of tat. Derivatives of the tat protein with substitutions either at the amino-terminus or at four cysteine residues were no longer able to transactivate HIV LTR-directed gene expression, although the protein retained its normal subcellular localization in the nucleus. Incubation of tat with zinc (a metal ion required for tat function) demonstrated that both authentic tat and cysteine mutation derivatives could form metallo-protein complexes. Tat proteins containing alterations within a cluster of positively charged amino acids retained their ability to transactivate gene expression, but at markedly reduced levels. Indirect immunofluorescence demonstrated that the tat protein and the amino-terminal and cysteine substitution mutants all were localized in the nucleus, with accumulation being most evident in the nucleolus. This suggests that the small 14 kD tat protein is a complex protein with at least three distinct functional domains. These results should aid in understanding the mechanism of transactivation and may provide information for the design of agents to inhibit HIV replication (10,11).

The lack of a suitable small animal model has prevented the direct in vivo analysis of HIV infections; consequently, few details are known of various stages of viral pathogenesis, including infection, virus replication, latency and disease progression. Homozygous SCID (severe combined immunodeficiency) mice, genetically immunodeficient due to their lack of functional T- and B-cells, are unable to mount an effective cellular or humoral immune response to foreign antigens. Dr. John Brown and collaborators have demonstrated that an implant of human lymphoid tissue, either fetal thymus glands or lymph nodes or both, together with human fetal liver hematopoietic cells, will support the differentiation and maturation of human T- and B-cells after transplant into SCID mice (SCID-hu mice). (It should be noted that all fetal tissues are obtained from therapeutic procedures not related to the NCI-funded research efforts). Fetal liver serves as a source of human stem cells, while the human thymus and lymph nodes provide the appropriate environment for stem cells to mature into functional immune cells. These transplanted lymphoid organs present full complements of functional and interactive cells which are permissive for HIV infection and have microscopic features and cellular compositions that are indistinguishable from normal human tissues. Since the SCID-hu mice, containing the transplanted lymphoid organs, express a transient wave of human $CD4^+$ and $CD8^+$ T-cells which are susceptible to HIV infection, these mice have been utilized as a small animal model for the study of the interactions of HIV with various components of the human lymphoid system. Eight weeks after tissue transplant, SCID-hu mice were inoculated with cloned HIV-1. In a time- and dose-dependent fashion virus replication spread within the transplanted human lymphoid organs. Immunohistochemistry and in situ hybridization revealed only viral RNA transcripts in most infected cells, while some cells had both detectable viral transcripts and viral proteins. This small animal model may therefore provide an appropriate system to systematically dissect cellular and molecular interactions involved in HIV pathogenesis (RNA I component, reference 12).

All viruses of the lentivirus subfamily consist of dense cylindrical nucleoids containing viral RNA, core proteins and reverse transcriptase. The viral genome codes for structural virion proteins and envelope glycoproteins, nonstructural proteins such as the viral polymerase and protease, and four or five other proteins involved in regulation of viral gene expression. Research in the laboratory of Dr. Max Essex has led to the discovery of proteins, unique to HIV-1 and HIV-2, which may provide reagents to permit a determination of the presence of the different types of HIV. An open reading frame, termed orf-u, was discovered within the HIV-1 genome with the potential to encode an additional viral protein of about 80 amino acids. Antibody to such a protein was detected in the sera of AIDS patients; however, unlike antibody to some other HIV-1 proteins, the prevalence of antibody to orf-u is significantly elevated in these patients. Because no analogous coding region has been identified in HIV-2, the antibody to orf-u may serve as a marker to distinguish HIV-1 and HIV-2 infections. Dr. Essex also demonstrated the existence of an additional open reading frame, orf-x, in HIV-2 and SIV but not in HIV-1. His results indicate that the orf-x products of SIV and HIV-2 are associated with cell-free virions and that there is protein sequence conservation of orf-x between HIV-2 and SIV, as deduced from immunologic cross reactivity. Antibody reactivity to the orf-x product was detected in 35 of 42 HIV-2-positive serum samples and 11 of 52 SIV-seropositive monkeys; however, no such antibodies were detected in HIV-1-positive donors, blood donors seronegative for both HIV-2 and HIV-1, or SIV-seronegative monkeys. Although the functions of the HIV-1 orf-u and HIV-2 orf-x proteins are presently unknown,

these proteins and antibodies to them should be useful to researchers and clinicians in distinguishing HIV-1 and HIV-2 infections (5).

AIDS is associated with an increased incidence of neoplasia, such as malignant lymphomas and Kaposi's sarcoma. Information relating to the natural history of AIDS-associated neoplasias was obtained from clinical and pathology records for 1981 through 1986; 105 patients with lymphoid neoplasia associated with AIDS, AIDS related complex, or at increased risk for AIDS were identified. Eighty-nine of these patients were diagnosed with non-Hodgkin's lymphoma, 13 presented Hodgkin's disease, and 3 had chronic lymphocytic leukemia. Immunologic analysis demonstrated a B-cell origin of the non-Hodgkin's lymphomas and the clonal suppressor-cytotoxic T-cell subset origin of the chronic lymphocytic leukemias; 69% of the non-Hodgkin's lymphomas were classified as high grade (small, noncleaved and large cell, immunoblastic-plasmacytoid) and 31% as intermediate grade (diffuse large cell). Each histopathologic category was correlated with distinct clinical features, including a statistically significant difference in median survival time. Patients with Hodgkin's disease demonstrated an atypical, aggressive clinical disease course, whereas patients with T-cell chronic lymphocytic leukemia had a more indolent clinical course. These retrospective studies demonstrated the clinical, morphologic and immunophenotypic spectrum of AIDS-associated lymphoid neoplasias, and indicate that the natural history of Hodgkin's disease is altered in patients with AIDS (1).

Investigators supported by the AIDS Virus Studies component have made progress on a number of aspects of HIV pathogenesis, including new insights into the interaction of the virus with cellular receptors, studies of pathogenesis in a mouse model, the existence of HIV as a population of viruses with varying degrees of pathogenicity and the demonstration of antigens useful in the differentiation of HIV-1 from HIV-2. This research has advanced our knowledge of the functions of HIV viral genes, the use of animal models for investigations of HIV pathogenesis and the association of HIV and AIDS with neoplastic sequelae and other diseases. However, additional studies are required to understand the mechanism of HIV pathogenesis with the overall goal of preventing HIV disease and treatment of those individuals already infected.

AIDS VIRUS STUDIES

GRANTS ACTIVE DURING FY89

Investigator/Institution/Grant Number	Title
1. DALLA-FAVERA, Riccardo Columbia University 2 R37 CA 37295-06	AIDS-Associated Lympho-proliferative Disorders
2. DAVIS, William C. Washington State University 1 R01 CA 50141-01	Animal Models for Research on AIDS-Related Lentiviruses
3. DOUGHERTY, Joseph P. Robert Wood Johnson Medical School (Piscataway, NJ) 1 R29 CA 50777-01	Determination of Retrovirus Mutation Rates
4. ELDER, John H. Scripps Clinic and Research Foundation 5 R01 CA 43362-04	Development of a Synthetic Vaccine to Retroviruses
5. ESSEX, Myron E. Harvard University 5 R35 CA 39805-05	NCI Outstanding Investigator Grant
6. FINBERG, Robert W. Dana-Farber Cancer Institute 5 R01 CA 34979-06	Animal Models of AIDS
7. FOX, C. Fred University of California, Los Angeles 1 R13 AI/CA 28033-01	Conference on Human Retroviruses
8. GRANT, Christopher K. Pacific Northwest Research Foundation 5 R01 CA 43371-04	Anti-Idiotype Vaccines for Feline Leukemia Virus
9. GREEN, William R. Dartmouth College 2 R01 CA 43475-04	Study on Development and Assessment of Retroviral Vaccines
10. HASELTINE, William A. Dana-Farber Cancer Institute 5 R01 CA 42098-03	Molecular Biology of the AIDS Virus HTLV-III
11. HASELTINE, William A. Dana-Farber Cancer Institute 5 R01 CA 44460-03	Molecular Biology of the ART Gene of HTLV-III

12. HIRSCH, Martin S.
Massachusetts General Hospital
2 R01 CA 12464-19
Immune Reactivity and Oncogenic Virus Infections

13. HIRSCH, Martin S.
Massachusetts General Hospital
5 R01 CA 35020-07
Viruses, Acquired Immunodeficiency, and Kaposi's Sarcoma

14. HOOVER, Edward A.
Colorado State University
2 R01 CA 43216-04
Pathogenesis of Feline Leukemia Virus Induced AIDS

15. LETVIN, Norman L.
Harvard University
1 R01 CA 50139-01
Immune Regulations in SIV Infections

16. LI, Yen
Harvard University
1 R01 CA 50146-01
Diversity and Pathogenesis of SIVagm

17. MONTELARO, Ronald C.
Louisiana State University
1 R01 CA 49296-01
Gene Expression During Lentivirus Infections: EIAV

18. NELSON, Jay A.
Scripps Clinic and Research Foundation
1 R01 CA 50151-01
A Transgenic Model for HIV/Opportunistic Interactions

19. NEURATH, A. Robert
New York Blood Center
5 R01 CA 43315-03
Synthetic HTLV-III Env Protein Analogs for Future Vaccines

20. PEDERSEN, Niels C.
University of California, Davis
1 R01 CA 50179-01
Incidental Infectious Diseases as Cofactors in the Transmission and Progression of FIV Infection

21. PITHA-ROWE, Paula M.
Johns Hopkins University
1 R01 CA 50158-01
Retrovirus-Induced Immunodeficiency: Role of Cytokines in Pathogenesis

22. ROTH, James A.
Iowa State University
1 R01 CA 50159-01
Bovine Lentivirus as a Model for HIV Infection

23. SCHOOLEY, Robert T.
Massachusetts General Hospital
5 R01 CA 37461-06
Cellular Immune Response to HIV

24. VOLSKY, David J.
St. Luke's-Roosevelt Hospital Center
5 R01 CA 43464-04
Novel Retroviruses from South America: HTLV-Type Viruses

CONTRACTS ACTIVE IN FY89

Investigator/Institution/Contract Number	Title
25. STABINSKY, Yitzhak TBC Research Laboratories N43 CP 95668	Enhanced Production of HIV-1 Protease in E. coli
26. WORDEN, Margaret Verax Corporation N43 CP 95669	Production of HIV gp120 Protein by Recombinant HeLa Cells in Collagen Microspheres

SUMMARY REPORT

RESEARCH RESOURCES

The Research Resources component of the Biological Carcinogenesis Branch (BCB), in conjunction with the various research units of the Branch, is responsible for developing, allocating and maintaining a coordinated program of research material support to meet the needs of extramural investigators funded by the Branch. The planning, initiating, and oversight necessary to generate and maintain specific research resources is the responsibility of the individual Program Directors who administer each of the research components of the Branch. However, the storage and distribution of research materials, the management of some resource contracts, the development and maintenance of a computerized inventory, and the day-to-day general management and direction of all resources distribution are the responsibility of the Research Resources component of the Branch. Currently four research resources contracts are administered by the Branch with an estimated FY 1989 funding level of 0.66 million dollars.

Laboratory investigations carried out under the sponsorship of the BCB depend on the availability of adequate quantities of viruses, viral reagents, antisera, animals, and clinical and laboratory materials of adequate purity, viability and potency, some of which are not available from the commercial sector. The BCB resources component provides some research materials and other supporting activities through contract operations in four general areas. These include activities directed toward production, characterization and distribution of viral and anti-viral reagents; activities concerned with animal resources, including breeding and maintenance of animal colonies; activities directed toward the provision of specialized testing services for the examination of experimental materials; and activities concerned with the storage, inventory and distribution of human specimens.

A consistently active supply of avian myeloblastosis virus (AMV) reverse transcriptase is vital to biological carcinogenesis studies involving the production of cDNA copies of retrovirus genomes for use as probes to identify viral sequences in normal or malignant tissues, to compare viral and cellular sequences for homology, to permit expression of viral sequences in bacterial systems and for other molecular biological studies. To meet these needs, more than 1,000,000 units of AMV reverse transcriptase were produced and over 190 shipments were made to domestic and foreign laboratories. Demand for this reagent has continued at a high level. However, due to the increasing availability of AMV reverse transcriptase from commercial sources, the contract for production of this reagent will not be continued beyond the current contract period (3).

In the search for oncogenic viruses, many cell cultures from the same or different species are used concurrently, thus offering frequent opportunities for cross-contamination. In cross-species tumor transplantations, it is important to be able to determine the derivation of induced tumors. Additionally, the significance of the presence of virus in tissue cells, the ability to grow virus, or the validity of virus isolation systems are all dependent upon the assurance of the identity of the cell cultures used. To meet this need, a maximum of three assays were carried out on approximately 375 cultures from over 50 laboratories. In making these interspecies and intraspecies cell identifications, more than 1,000 procedures were performed using the following assays: immunofluorescent

staining for species-specific surface antigens, isoenzyme analysis, and cytological analysis by means of chromosome banding (4).

During this period, more than 300 shipments of viral reagents and human specimens were made to domestic laboratories from the inventory of frozen biological reagents. Appropriate demographic, clinical and characterization data were included with each shipment. In addition, over 30 shipments of reagents and data were sent to foreign laboratories (2).

Additionally, each year the Branch coordinates and prepares for publication an advertisement of resources and services available for cancer research from all programs within the Division of Cancer Etiology (DCE). This advertisement, which keeps the scientific community advised of currently available resources under the auspices of DCE and the contractor source of supply, is published in several major scientific journals each year.

All resource contracts administered by the Branch operate under a payback system which was implemented in 1981. Under this system, some of the costs of production, along with the costs for handling and distribution of research resources are collected from the recipient. The payback system seems to be performing as expected. The demand level for high quality biological reagents not readily available from commercial sources has remained fairly constant. Reimbursement for full or partial costs of services has led to more careful use of costly resource reagents. This has resulted in a reduced level of effort in several resource contracts or the termination of activities deemed to be no longer necessary.

RESEARCH RESOURCES

CONTRACTS ACTIVE DURING FY89

Investigator/Institution/Contract Number	Title
1. CLAPP, Neal K. Oak Ridge Associated Universities NO1 CP 51006	Operation of a Marmoset Colony for Cancer Research
2. DONLEY, Elizabeth Microbiological Associates Inc. NO1 CP 61020	Repository for Storage and Distribution of Viruses, Viral Reagents and Human Sera
3. HOUTS, G. E. Molecular Genetics Resources, Inc. NO1 CP 51007	Production, Characterization and Distribution of AMV Reverse Transcriptase
4. PETERSON, Ward D. Children's Hospital of Michigan (Detroit) NO1 CP 8564	Cell Culture Identification and Cytologic/Karyotypic Analysis